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**BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES**

Paper No. 68

Application Number: 08/966,233

Filing Date: November 7, 1997

Appellant(s): LEE, SE-JIN

Bonnie Weiss McLeod
For Appellant

EXAMINER'S ANSWER

This is in response to the appeal brief filed 5/14/03.

(1) *Real Party in Interest*

A statement identifying the real party in interest is contained in the brief.

(2) *Related Appeals and Interferences*

A statement identifying the related appeals and interferences which will directly affect or be directly affected by or have a bearing on the decision in the pending appeal is contained in the brief.

(3) *Status of Claims*

The statement of the status of the claims contained in the brief is incorrect. A correct statement of the status of the claims is as follows:

Claims 1-21 were presented with the application as filed. Claims 22-23 were added in the amendment filed 1/5/96 (amendment F, Paper No. 28). Claims 24-31 were added in the amendment filed 11/7/97 (amendment I, Paper No. 39). Claims 32-38 were added in the amendment filed 4/3/01 (amendment L, Paper No. 52). Claims 39-42 were added in the amendment filed 10/19/01 (amendment M, Paper No. 55). Claims 1, 4-10, and 16-21 were cancelled in the amendment filed 4/15/94 (amendment C, Paper No. 17). Claim 2 was cancelled in the amendment filed 1/5/96 (amendment F, Paper No. 28). Claim 23 was cancelled in the amendment filed 11/7/97 (amendment I, Paper No. 39).

Claims 3, 11-15, 22, and 24-42 are pending and on appeal.

(4) *Status of Amendments After Final*

The appellant's statement of the status of amendments after final rejection contained in the brief is correct. The amendment after final rejection submitted 3/21/03 (amendment N, Paper No. 62) has not been entered.

(5) *Summary of Invention*

The summary of invention contained in the brief is deficient.

The brief asserts at page 3, lines 1-3, that the two other clones found in the library are "believed to represent allelic variations within the gene." This misrepresents the text at page 19, lines 17-29, of the specification. The specification states the "changes may represent allelic differences or they may indicate the presence of multiple GDF-1 genes."

(6) *Issues*

The appellant's statement of the issues in the brief is substantially correct. The changes are as follows:

Claims 3, 11-15, 22, and 24-42 are rejected under 35 USC 101 and 112 with respect to utility and how to use the claimed invention.

Claims 3, 11-15, 22, 24-34, and 39-42 are rejected under 35 USC 112 with respect to written description. Claims 39-42 were particularly addressed with respect to new matter; however, this was not a separate ground of rejection. In view of appellant's arguments, this rejection has been withdrawn with respect to claims 35-38.

(7) *Grouping of Claims*

The appellant's statement in the brief that certain claims do not stand or fall together is not agreed with.

Claims 3, 11-15, 22, 24-34, and 39-42 stand or fall together with regard to the utility and enablement (how to use) rejections set forth under 35 USC 101 and 112.

Appellant's brief includes a statement that claims 22, 3, and 11-15; claims 31-34 and 39-42; and claims 24-30 and 35-38 have been separated into three distinct groups with respect to the

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rejection under 35 USC 112, 1st paragraph, for written description; however, the brief fails to provide reasons as set forth in 37 CFR 1.192(c)(7) and (c)(8). In view of appellant's arguments, this rejection has been withdrawn with respect to claims 35-38; however, there is a single ground of rejection under 35 USC 112, 1st paragraph, for written description and claims 3, 11-15, 22, 24-34, and 39-42 stand or fall together.

(8) *ClaimsAppealed*

A substantially correct copy of appealed claim 22 on page 23 (Exhibit A) of appellant's brief. The minor errors are as follows: In claim 22, line 1, "having the amino" should be – having an amino--. See amendment filed 11/7/97 (amendment I, Paper No. 39).

(9) *Prior Art of Record*

Copies of all references are attached to this document.

Akhurst, R. J. et al. "Transforming Growth Factor Betas in Mammalian Embryogenesis," *Progress in Growth Factor Research*, Volume 2, pages 153-168, 1990.

Bengtsson, H. et al. "Potentiating Interactions Between Morphogenetic Protein and Neurotrophic Factors in Developing Neurons," *Journal of Neuroscience Research*, Volume 53, pages 559-568, 1998.

Ebendal, T. et al. "Glial Cell Line-Derived Neurotrophic Factor Stimulates Fiber Formation and Survival in Cultured Neurons From Peripheral Autonomic Ganglia," *Journal of Neuroscience Research*, Volume 40, pages 276-284, 1995.

Ebendal, T. et al. "Bone Morphogenetic Proteins and Their Receptors: Potential Functions in the Brain," *Journal of Neuroscience Research*, Volume 51, pages 139-146, 1998.

Ermfors, P. et al. "Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: Developmental and topographical expression in the brain," *Proceedings of the National Academy of Sciences*, Volume 87, pages 5454-5458, July 1990.

Hoban, C. J. et al. "Activation of Second Messenger Pathways by GDF-1," *Society for Neuroscience Abstracts*, volume 19, page 653, Abstract 275.9, November 7-12, 1993.

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Kriegstein, K. et al. "Distinct Modulatory Actions of TGF- β and LIF on Neurotrophin-Mediated Survival of Developing Sensory Neurons," *Neurochemical Research*, Volume 21, Number 7, pages 843-850, 1996.

Massague, J. "The Transforming Growth Factor - β Family," *Annual Review of Cell Biology*, Volume 6: pages 597-641, 1990.

Rankin, C. T. et al. "Regulation of left-right patterning in mice by growth/differentiation factor-1," *Nature Genetics*, Volume 24, pages 262-265, March 2000.

(10) Grounds of Rejection

The following ground(s) of rejection are applicable to the appealed claims:

Claims 3, 11-15, 22, and 24-42 are rejected under 35 U.S.C. § 101 because the claimed invention lacks patentable utility due to its not being supported by either a specific, substantial, and credible utility or by a well established utility.

Claims 3, 11-15, 22, and 24-42 are also rejected under 35 U.S.C. § 112, first paragraph. Specifically, since the claimed invention is not supported by either a specific, substantial, and credible asserted utility or a well established utility, one skilled in the art would not know how to use the claimed invention.

Claims 3, 22, 24-25, 32, and 35 are directed to isolated DNA segments encoding GDF-1 proteins. Claim 31 is directed to a complementary DNA segment. Claims 11, 26, and 33 are directed to vector containing a DNA segment encoding GDF-1. Claims 12-14, 27-29, and 36 are directed to host cells. Claims 15, 30, 34, and 37 are directed to methods of producing recombinant GDF-1. Claims 39-42 are directed to DNA segments encoding GDF-1 proteins where the sequences hybridize under particular conditions, vectors, host cells, and methods of production, respectively. The protein products lack patentable utility for the reasons set forth

below; therefore, the methods of producing the protein and vectors and hosts used therefore to make these protein products must also lack patentable utility.

The specification and claims disclose using the nucleic acid sequences encoding GDF-1 to produce GDF-1 proteins.

The specification discloses using the GDF-1 proteins to make antibodies. (See specification page 12, lines 3-7.) This is not considered to be a specific asserted utility because it is generally applicable to any protein.

The specification discloses a general expectation that GDF-1 will “likely play an important role in mediating developmental decisions related to cell differentiation.” (See specification page 2.) This expectation is premised upon the structural similarity of GDF-1 to members of the TGF- β superfamily. The similarities between murine GDF-1 and some TGF- β family members ranges from 26-52% in the region starting with the first conserved cysteine and extending to the C-terminus. GDF-1 is most homologous to Vg-1 and least homologous to inhibin- α . The specification also discloses that GDF-1 is not homologous outside this region to Vg-1 and acknowledges that murine GDF-1 is not the murine homolog of Vg-1. (See specification page 20.) Note that this similarity determination is only for approximately 107 out of 357 amino acids and not for the full length amino acid sequence. (See page 19 in view of the sequence in Figure 2A-B and alignments in Figure 3A.) However, the specification fails to demonstrate or specifically associate any particular activity with GDF-1 proteins, and the specification discloses that the activities of the members of the TFG- β superfamily vary quite widely. (See specification at pages 1-2 and 12-15.) The specification states on page 12 that a potential use for GDF-1 is as a diagnostic tool as a specific marker for the presence of tumors

arising from cell types that normally express GDF-1. Other disclosed potential uses are as an indicator for developmental anomalies in prenatal screens for birth defects or genetic diseases. However, no tumors are identified for any cell types that normally express GDF-1. As such, experimentation would be required to establish or reasonably confirm that it could be used as a specific marker for any tumor. Likewise, the specification does not associate GDF-1 with any birth defects or genetic diseases and experimentation would be required to establish or reasonably confirm that it could be used in this manner. Rather, the specification discloses **potential activities and potential uses** if one or another activity should be associated with GDF-1 when the protein is further characterized. For example, the specification at page 13, lines 16-24, states, “**Potential** uses for GDF-1 as a therapeutic tool are also suggested by the known biological activities of the other members of this superfamily. For example, since some of these proteins act as cell-specific growth inhibitors, one **potential** therapeutic use for GDF-1 is as an anti-cancer drug to inhibit the growth of tumors derived from cell types that are normally responsive to GDF-1” (emphasis added). However, the specification describes no tumors associated with GDF-1 nor any cells that are normally responsive to GDF-1. Indeed, the following paragraphs in the specification describe “converse” or “alternative” activities. For example, the specification at page 13, lines 33-35, states, “**Conversely**, if GDF-1 functions as a growth-stimulatory factor for specific cell types, other **potential** therapeutic uses will be apparent” (emphasis added). Notice that these are diametrically opposed activities and that these uses are predicated upon further experimentation to characterize the protein. The necessity for such experimentation is stated within the specification itself at page 14, lines 29-33. “A determination of the specific clinical settings in which GDF-1 will be used as a diagnostic or as a

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therapeutic tool **await further characterization** of the expression patterns and biological properties of GDF-1 both under normal physiological conditions and during disease states” (emphasis added).

The claimed GDF-1 proteins are not supported by a substantial utility as the specification makes clear that further experimentation is necessary to determine and/or confirm the activity and uses of the protein. Identifying and studying the properties of a protein itself or the mechanisms in which the protein is involved does not define a "real world" context or use. The specification does not inform those skilled in the art how to use the claimed invention with any particularity. The specification is required to clearly state how the claimed invention is to be used. It should be apparent to one of ordinary skill in the art how the claimed invention is to be used after reading the specification. One of ordinary skill in the art should not have to envision, infer, or "dream up" potential uses or perform undue experimentation to determine how to use the claimed invention. That is, the specification is an invitation to experiment to determine how to use GDF-1. This specification is analogous to that in Genentech Inc. v. Novo Nordisk A/S, 42 USPQ2d 1001, 1005, which was not deemed to be enabling. "It is the specification, not the knowledge of one skilled in the art, that must supply the novel aspects of an invention in order to constitute adequate enablement. This specification provides only a starting point, a direction for further research." The specification speculates on possible activities of GDF-1. None of the particular activities disclosed for other TGF- β superfamily members have been demonstrated for this protein in the specification and none were known at the time of the invention. None of the uses set forth in the specification could be practiced at the time of the invention without undue

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experimentation. Providing a laundry list of potential uses, some of which are diametrically opposed to each other, is not deemed to be enabling.

The relevant portion of pages 1-2 of the specification are reproduced below.

15 A growing number of polypeptide factors
 playing critical roles in regulating differentiation
 processes during embryogenesis have been found to be
 structurally homologous to transforming growth
 factor β (TGF- β). Among these are Mullerian
20 inhibiting substance (MIS) [Cate et al, Cell 45:685-
 698 (1986)], which causes regression of the
 Mullerian duct during male sex differentiation; the
 bone morphogenetic proteins (BMP's) [Wozney et al,
 Science 242:1528-1534 (1988)], which can induce de
25 novo cartilage and bone formation; the inhibins and
 activins [Mason et al, Nature 318:659-663 (1985);
 Forage et al, Proc. Natl. Acad. Sci., USA 83:3091-
 3095 (1986); Eto et al, Biochem Biophys Res Comm
 142:1095-1103 (1987); and Murata et al, Proc. Natl.
30 Acad. Sci. USA 85:2434-2438 (1988)], which regulate
 secretion of follicle-stimulating hormone by
 pituitary cells and which, in the case of the
 activins, can affect erythroid differentiation; the

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Drosophila decapentaplegic (DPP) gene product [Padgett et al, Nature 325:81-84 (1987)], which influences dorsal-ventral specification as well as morphogenesis of the imaginal disks; the Xenopus 5 Vg-1 gene product [Weeks et al, Cell 51:861-867 (1987)], which localizes to the vegetal pole of eggs; and Vgr-1 [Lyons et al, Proc. Natl. Acad. Sci., USA 86:4554-4558 (1989)], a gene identified on the basis of its homology to Vg-1 and shown to be 10 expressed during mouse embryogenesis. In addition, one of the most potent mesoderm-inducing factors, XTC-MIF, also appears to be structurally related to TGF- β [Rosa et al, Science 239:783-785 (1988); and Smith et al, Development 103:591-600 (1988)]. The 15 TGF- β 's themselves are capable of influencing a wide variety of differentiation processes, including adipogenesis, myogenesis, chondrogenesis, hematopoiesis, and epithelial cell differentiation [Massague, J., Cell 49:437-438 (1987)], and at least 20 one TGF- β , namely TGF- β 2, is capable of inducing mesoderm formation in frog embryos [Rosa et al, Science 239:783-785 (1988)].

The present invention relates to a new member of the TGF- β superfamily, and to the 25 nucleotide sequence encoding same. This new gene and the encoded protein, like other members of this superfamily, are likely play an important role in mediating developmental decisions related to cell differentiation.

30

SUMMARY OF THE INVENTION

It is a general object of the present invention to provide a novel cell differentiation regulatory factor and a nucleotide sequence encoding same.

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The relevant portion of pages 12-15 of the specification are reproduced below.

The TGF- β superfamily encompasses a group of proteins affecting a wide range of differentiation processes. The structural homology between GDF-1 and the known members of the TGF- β superfamily and the pattern of expression GDF-1 during embryogenesis indicate that GDF-1 is a new member of this family of growth and differentiation factors. Based on the known properties of the other members of the this superfamily, GDF-1 can be expected to possess biological properties of diagnostic and/or therapeutic benefit in a clinical setting.

For example, one potential use for GDF-1 as a diagnostic tool is as a specific marker for the presence of tumors arising from cell types that normally express GDF-1. The availability of such markers would be invaluable for identifying primary and metastatic neoplasms of unknown origin or for monitoring the response of an identified neoplasm to a particular therapeutic regimen. In this regard, one member of this superfamily, namely, inhibin, has been shown to be useful as a marker for certain ovarian tumors [Lappohn et al, N. Engl. J. Med. 321:790 (1989)].

A second potential diagnostic use for GDF-1 is as an indicator for the presence of developmental anomalies in prenatal screens for potential birth defects. For example, abnormally high serum or amniotic fluids levels of GDF-1 may indicate the presence of structural defects in the

developing fetus. Indeed, another embryonic marker, namely, alpha fetoprotein, is currently used routinely in prenatal screens for neural tube defects [Haddow and Macri, JAMA 242:515 (1979)].

5 Conversely, abnormally low levels of GDF-1 may indicate the presence of developmental anomalies directly related to the tissues normally expressing GDF-1.

A third potential diagnostic use for GDF-
10 1 is in prenatal screens for genetic diseases that either directly correlate with the expression or function of GDF-1 or are closely linked to the GDF-1 gene. Other potential diagnostic uses will become evident upon further characterization of the
15 expression and function of GDF-1.

Potential uses for GDF-1 as a therapeutic tool are also suggested by the known biological activities of the other members of this superfamily. For example, since some of these proteins act as
20 cell-specific growth inhibitors, one potential therapeutic use for GDF-1 is as an anti-cancer drug to inhibit the growth of tumors derived from cell types that are normally responsive to GDF-1.

Indeed, one member of this superfamily, namely,
25 Mullerian inhibiting substance, has been shown to be cytotoxic for human ovarian and endometrial tumor cells either grown in culture [Donahoe et al, Science 205:913 (1979); Fuller et al, J. Clin. Endocrinol. Metab. 54:1051 (1982)] or when
30 transplanted into nude mice [Donahoe et al, Ann. Surg. 194:472 (1981); Fuller et al, Gynecol. Oncol. 22:135 (1984)].

Conversely, if GDF-1 functions as a growth-stimulatory factor for specific cell types, other potential therapeutic uses will be apparent.
35 For example, one member of this superfamily, namely, activin, has been shown to function as a nerve cell

survival molecule [Schubert et al, *Nature* 344:868 (1990)]. If GDF-1 possesses a similar activity, as is indicated by its specific expression in the central nervous system (see below), GDF-1 will likely prove useful in vitro for maintaining neuronal cultures for eventual transplantation or in vivo for rescuing neurons following axonal injury or in disease states leading to neuronal degeneration. Alternatively, if the target cells for GDF-1 in the nervous system are the support cells, GDF-1 will likely prove to be of therapeutic benefit in the treatment of disease processes leading to demyelination.

Many of the members of this superfamily, including GDF-1, are also likely to be clinically useful for tissue repair and remodeling. For example, the remarkable capacity of the bone morphogenetic proteins to induce new bone growth [Urist et al, *Science* 220:680 (1983)] has suggested their utility for the treatment of bone defects caused by trauma, surgery, or degenerative diseases like osteoporosis. Indeed, the bone morphogenetic proteins have already been tested in vivo in the treatment of fractures and other skeletal defects [Glowacki et al, *Lancet* i:959 (1981); Ferguson et al, *Clin. Orthoped. Relat. Res.* 227:265 (1988); Johnson et al, *Clin. Orthoped. Relat. Res.* 230:257 (1988)].

A determination of the specific clinical settings in which GDF-1 will be used as a diagnostic or as a therapeutic tool await further characterization of the expression patterns and biological properties of GDF-1 both under normal physiological conditions and during disease states. Based on the wide diversity of settings in which other members of this superfamily may be used for clinical benefit, it is likely that GDF-1 and/or

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antibodies directed against GDF-1, will also prove to be enormously powerful clinical tools. Potential uses for GDF-1 will almost certainly include but not be restricted to the types of clinical settings described above. Moreover, as methods for improving the delivery of drugs to specific tissues or to specific cells become available, other uses for molecules like GDF-1 will become evident.

The relevant portions of page 20 are reproduced below.

Figure 3b shows a tabulation of the percentages of identical residues between GDF-1 and the other members of the TGF- β family in the region starting with the first conserved cysteine and extending to the C-terminus. GDF-1 is most homologous to Vg-1 (52%) and least homologous to inhibin- α (22%) and the TGF- β 's (26-30%). Two lines of reasoning indicate that GDF-1 is not the murine homolog of Vg-1. First, GDF-1 is less homologous to Vg-1 than are Vgr-1 (59%), BMP-2a(59%), and BMP-2b (57%). Second, GDF-1 does not show extensive homology with Vg-1 outside of the C-terminal portion, and it is known that other members of this family are highly conserved across species throughout the entire length of the protein [Cate et al, Cell 45:685-698 (1986); Mason et al, Nature 318:659-663 (1985); Forage et al, Proc. Natl. Acad. Sci., USA 83:3091-3095 (1986); Derynck et al, Nature 316:701-705 (1985); Mason et al, Biochem. Biophys. Res. Comm. 135:957-964 (1986); and Derynck et al, J. Biol. Chem. 261:4377-4379 (1986)]. However, GDF-1 and Vg-1 do share two regions of limited homology N-terminal to the presumed dibasic cleavage site, as shown in Figure 3c.

When read fully and in context, the specification as filed does not set forth a utility that is specific, substantial, and credible. The specification as filed discloses the necessity for further experimentation to characterize and determine how to use the GDF-1 proteins. In view of the requirement for further experimentation, no well known utility can be considered to have been known for the GDF-1 proteins at the time of the invention.

Furthermore, the specification does not enable using GDF-1 in any capacity without undue experimentation. Again, the specification is an invitation to experiment without clear direction or guidance as to the particular biological activity to investigate. Embryogenesis and mediation of cell differentiation are broad areas of basic research. No tumors nor developmental defects are identified as being associated for any screening or diagnostic methods. No normal or abnormal levels for GDF-1 are disclosed in the specification for any cell type or tissue. No direction or guidance as to particular known tumors or known developmental defects to be investigated are provided.

Claims 3, 5-11, 22, 24-34, and 39-42 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Claims 39-42 include limitations where DNA hybridizes under conditions of 65 degrees Celsius and 1 M sodium chloride to DNA having the nucleotide sequence as defined in Figure 2 or Figure 11A or 11B and remains bound when subjected to washing at 68 degrees Celsius and

0.3 M sodium chloride/ 30 mM sodium citrate (2X SSC). The specification does not disclose these limitations and as such the claims embrace new matter.

Appellant has previously pointed to page 10 and 17 of the specification for basis; however, the portion relied upon does not disclose the limitations as presently claimed.

The referenced portion of page 10 is reproduced below.

The invention further relates to DNA segments substantially identical to the sequence shown in Figure 2. A "substantially identical" sequence is one the complement of which 5 hybridizes to the sequence of Figure 2 at 68°C and 1M NaCl and which remains bound when subjected to washing at 68°C with 0.1X saline/sodium citrate (SSC) (note: 20 x SSC = 3M sodium chloride/0.3 M sodium citrate).

Note that this disclosure is with respect to the sequence of Figure 2 alone and not Figure 11A or 11B and that the hybridization is at 68 degrees Celsius and not 65 degrees Celsius.

The referenced portion of page 17 is reproduced below.

For Southern analysis, DNA was 10 electrophoresed on 1% agarose gels, transferred to nitrocellulose, and hybridized in 1M NaCl, 50 mM sodium phosphate, pH 6.5, 2 mM EDTA, 0.5% SDS, 10X Denhardt's at 65°C. The final wash was carried out in 2X SSC at 68°C.

Note that this disclosure is in the context of a particular experimental technique and is not associated with the what was intended by the prior disclosure of "substantially identical sequences." It is not disclosed with respect to hybridization of particular sequences in the }

absence of electrophoresis and transfer to nitrocellulose. It is not disclosed with hybridization to the particular sequences of Figure 2 or Figure 11A or 11B. Furthermore, the claims have no limitations corresponding to 50 mM sodium phosphate, pH 6.5, 2 mM EDTA, 0.5% SDS, and 10x Denhardt's which are clearly integral to this disclosure of hybridization at 65 degrees Celsius. There is nothing that ties these two separate portions of the disclosure together to convey to one of ordinary skill in the art that the invention now claimed was originally contemplated. It is further noted that the only Southern analysis performed in the specification in Example 3 on page 22 and in Figure 5, does not identify the sequence of the probe used but it appears that it must have been from mouse and not both mouse and human as encompassed by the claims in view of the limitations to Figures 2, 11A, and 11B.

Independent claims 22, 24, 31, and 39 recite "DNA segment encoding mammalian GDF-1 protein." In addition, claim 22 recites "comprising an amino acid sequence defined in an open reading frame" (emphasis added). In addition, claim 24 recites "comprising a nucleotide sequence as defined in an open reading frame" (emphasis added). This does not require the full open reading frame for GDF-1 as in claim 35 but includes subsequences as small as a single amino acid or single nucleotide. In addition, the claim does not recite that the open reading frame be for GDF-1 as in claim 35. Claims 3 and 11-15 depend upon claim 22. Claims 25-30 depend upon claim 24. Claims 32-34 depend upon claim 31. Claims 40-42 depend upon claim 39.

First of all, a gene or genomic DNA sequence includes regions that precede and follow the coding region as well as intervening sequences (introns) between individual coding segments

(exons). The intron is DNA that is transcribed but removed from the transcript by splicing together the bordering exons. The gene or genomic DNA sequence encodes the protein product as the gene or genomic DNA sequence is capable of producing the protein product when expressed in an appropriate host cell under suitable conditions. Thus, the claim language "DNA segment encoding" encompasses genomic sequences for GDF-1. However, the specification discloses no genomic sequence or structure (characterization of introns/exons, etc.) for any GDF-1 protein. The specification discloses particular nucleotide sequences for murine GDF-1 and human GDF-1. These sequences were cloned from cDNA libraries and are disclosed as cDNA sequences. (See for example, Figures 2, 11A, and 11B as well as pages 15-16, 18, and 28.) A cDNA or complementary DNA is a single stranded DNA complementary to an mRNA synthesized from it by reverse transcription *in vitro*. It reflects the spliced version (introns removed) of the sequence. These sequences do not provide a description of the genomic sequence. The Southern blot experiments in Example 3 and Figure 5 use mouse, human, and hamster genomic DNA but demonstrate that even under high stringency hybridization conditions, additional bands were detected in addition to a predominant band and their sequence structure is not described. The specification clearly distinguishes them from partial digestion products. That is, multiple, different, genomic sequences were found in each of these species. Furthermore, the actual sequence is not disclosed nor is there a characterization of any intron/exon/leader/trailer structure for any of these genomic sequences.

Secondly, the language "mammalian GDF-1 protein" requires that one of ordinary skill in the art be able to identify when a nucleic acid encoding a protein within this family is found, particularly for claims encompassing genomic sequences and hybridizing sequences. There is no

limiting definition in the specification as to the structural (e.g. degree of sequence similarity or a particular motif) or functional characteristics (e.g. a specific biological activity) that define a "mammalian GDF-1 protein." No assays are disclosed to screen for mammalian GDF-1 protein activity. One of ordinary skill in the art at the time of the invention would not know when a DNA segment encoding a mammalian GDF-1 protein had been obtained with the exception of the specific and full length sequences for mouse and human GDF-1.

As such, the claims embrace sequences that do not meet the written description provision of 35 USC 112, first paragraph. The specification provides insufficient written description to support the genus encompassed by the claims.

(11) *Response to Argument*

Appellant argues in the brief at page 7 that the predicted role of GDF-1 in embryogenesis is supported by the fact that other members of the TGF- β superfamily known at the time had a role in embryonic processes. Appellant relies upon Akhurst et al. However, Akhurst et al. discloses information about TGF- β 1, TGF- β 2, and TGF- β 3 in mammalian embryogenesis. Akhurst et al. also discusses the wide variety of activities found in the larger TGF- β superfamily. Page 155 states, "As yet there is no definitive evidence that any of the TGF β s are endogenous regulators of mammalian embryonic processes." It is emphasized by the examiner that what is under discussion in Akhurst et al. is TGF- β itself and not the larger superfamily. Thus, this reference provides no evidence with respect to the proteins of the larger superfamily and their role in mammalian embryogenesis. Appellant is reminded that the protein disclosed to have the highest homology to GDF-1 was not TGF- β 1, TGF- β 2, or TGF- β 3, but rather Vg-1 which is from amphibians and not mammals. The totality of Akhurst et al. fairly indicates that those of

skill in the art at the time of the invention were experimenting and looking to see whether TGF- β 1, TGF- β 2, and TGF- β 3 proteins were involved in mammalian embryogenesis and how. The conclusion and prospects section of the reference on pages 164-165 states that the evidence would suggest that each isoform of TGF- β (i.e. TGF- β 1, TGF- β 2, and TGF- β 3) has a distinct function *in vivo*. The reference states, “To test this proposition, it is essential that more functional studies are carried out.” This supports the examiner’s position that further research would be required to reasonably determine or confirm any activity or involvement of GDF-1 in embryogenesis. Furthermore, the reference amply illustrates that embryogenesis is a highly diverse and complex process including skeletal development, hematopoiesis, vascularization, and so forth. (See pages 157-164.) This is also acknowledged by the specification as filed on page 2, lines 15-20. As such, a disclosure that GDF-1 may be involved in embryogenesis cannot be considered to convey to those of ordinary skill in the art any specific or clear biological activity. It provides no direction or guidance as to which aspect or to a particular activity.

Appellant refers to the utility guidelines with respect to assignment of GDF-1 to a class of sufficiently conserved proteins that imputes the same specific, substantial, and credible utility to the assigned protein. First of all, the TGF- β superfamily has many subsets as evidenced by at least the specification and Akhurst et al. Secondly, the activities of the different members are diverse as evidenced by at least the specification and Akhurst et al. Third, the specification fails to assert a particular biological activity within the complex and diverse activities that “embryogenesis” embraces. To the degree that appellant is relying upon the statement at page 2, lines 25-29, concerning possibly “mediating developmental decisions related to cell differentiation” rather than embryogenesis generally, this statement is not clearly limited to

embryogenesis nor is it specific as to what type of mediation, what type of cells, and/or what type of differentiation. This is analogous to the fact pattern in *In re Kirk*, 153 USPQ 48, 52 in which nebulous expressions of biological activity in the specification did not convey a sufficiently explicit indication of the usefulness of the compounds and how to use them.

While appellant stresses the structural similarity of GDF-1 to members of the TGF- β superfamily, they are reminded that this similarity determination is only for approximately 107 out of 357 amino acids and not for the full length amino acid sequence. (See page 19 in view of the sequence in Figure 2A-B and alignments in Figure 3A.) They are further reminded that the specification also discloses that GDF-1 is not homologous outside this region to Vg-1. While the specification discloses that GDF-1 is most homologous to Vg-1 and least homologous to inhibin- α , no where in the specification is there an assertion that GDF-1 is expected to act similarly to a particular member of the superfamily. No where in the specification is there an assertion that GDF-1 is expected to act like the protein disclosed to have the highest similarity, Vg-1. In fact, the specification discloses that GDF-1 could have activities similar to members of the superfamily that are less structurally similar.

Appellant is reminded that the utility guidelines are just that, guidelines. They do not supersede the statutes nor the pertinent case law. In addition, the fact pattern here differs from those set forth in the utility guidelines but closely matches the fact pattern in the pertinent case law, *In re Kirk*, 153 USPQ 48. The disclosure of the originally filed specification does not provide a specific, substantial, and credible asserted utility nor a well established utility for the claimed invention.

Appellant relies upon the Ebendal declaration previously submitted under 37 CFR 1.132. The declaration supports the examiner's position that GDF-1 activity at the time of the invention was not known, could not have been predicted, and that there was no specific or substantial utility set forth as evidenced by the degree of experimentation conducted to find an activity.

This declaration sets forth that recombinant human GDF-1 (amino acids 255-373 fused to 34 additional amino acids) was produced in E. coli and recovered as a dimer. This product potentiates human NT-3 fibre outgrowth. The assays used to establish this biological activity are referenced to Ebendal (1995) and Ernfors (1990). (Note that these references were attached to the Ebendal declaration but have not been made of record.) The declaration asserts that this biological activity on neurons is similar to other members of the TGF- β superfamily.

First of all, the particular material tested is not disclosed in the specification. That is, while Figure 11B discloses the human GDF-1 sequence, the portion of this protein and the particular fusion partner used in the declaration experiments do not appear to be disclosed in the specification. Use of the particular pRSET vector by Invitrogen does not appear to be disclosed in the specification. Use of a dimer versus a monomer does not appear to be disclosed in the specification. The fibre outgrowth assay of Ebendal et al. (1995) was developed after the effective filing date of the application. The Ernfors et al. (1990) reference is also post-filing date for the ultimate parent application (07/538,372, filed 6/15/90). Furthermore, it discloses fibre outgrowth activity of NT-3 (although not named as such in this reference) but does not disclose similar activity of TGF- β superfamily members or GDF-1 proteins. It is noted that the declaration evidence indicates that GDF-1 alone was ineffective to evoke fibre outgrowth.

The potentiating activity between the TGF- β superfamily member OP-1 and NT-3 was not known until well after the effective filing date. (See Bengtsson et al., Journal of Neuroscience Research, 1998.) It is noted that the receptors discussed were not known at the time of the invention nor does the reference generally postulate this activity to all other members of the superfamily. The involvement of the GDF family was only determined well after the effective filing date. (See Ebendal et al., Journal of Neuroscience Research, 1998.) It was not discovered until well after the effective filing date that TGF- β 3 potentiates the survival achieved with NT-3 and NT-4. (See Kriegstein et al., Neurochemical Research, 1996.)

Massague provides a review of the TGF- β superfamily at approximately the time of the invention. The reference sets forth the diverse effects of the various members of the superfamily. Notably, the potentiating effect of the Ebendal declaration is not disclosed for any member of the superfamily.

As such, the Ebendal declaration cannot be considered to demonstrate that GDF-1 possesses a function predicted by the specification. Potentiation of human NT-3 fibre outgrowth by recombinant human GDF-1 (amino acids 255-373 fused to 34 additional amino acids) produced in E. coli and recovered as a dimer was not predicted by the specification. The function established was discovered in view of further research not contemplated by the specification and using materials and techniques not available at the time of the invention.

Appellant also relies upon the Rankin et al. reference. Appellant is relying upon the filing date of the ultimate parent application, 07/538,372, filed 6/15/90. The Rankin et al. (March 2000) was published well after the effective filing date of the instant invention and the abstract itself admits that the function of GDF-1 was not known when discovered by inventor

Lee. (See abstract citations 2 and 3.) It is noted that knockout mice were not routinely produced at the time of the invention. The specification does not predict that GDF-1 was involved in development of the left-right axis in mice and expression of genes expressed downstream in development. The specification does not disclose nor contemplate knockout mouse experimental models.

Like the Petrow affidavit in *In re Kirk*, 153 USPQ 48, 52, the specific biological activities discussed in the Ebendal declaration and Rankin et al. reference are absent from the specification disclosure. The Ebendal declaration and Rankin et al. reference amount to an admission that experimentation would be necessary to determine the actual uses.

Not only does the specification fail to inform one of ordinary skill in the art what to do with GDF-1, the specification cannot be considered to enable one to use GDF-1 in any of these later discovered contexts as each required experimentation well beyond the disclosure in the specification. The Ebendal declaration and Rankin et al. reference use information, materials, assays, and/or techniques that were not known at the time of the invention and thus make clear that one of ordinary skill in the art trying to determine what activity GDF-1 had at the time of the invention would have been required to go beyond routine experimentation.

It is noted that the Ebendal declaration and Rankin et al. do not speak to using GDF-1 or any other TGF- β superfamily member in prenatal screening for developmental defects in mice, humans, or any other species.

Finally, Hoban et al. (1993) establishes that even several years after the effective filing date of the invention the biological activity of GDF-1 was just being determined and assays for GDF-1 were just being developed. This abstract expressly states, "We have been working

towards identifying bioassays for GDF-1." The only results disclosed are that human GDF-1 stimulates immediate early gene expression in neural cell lines. Nothing in the specification would lead one of ordinary skill in the art to this activity or use.

Appellant argues on page 12 of the brief that Figure 7 shows that GDF-1 is expressed almost exclusively in brain. This is a misrepresentation. Northern analysis demonstrated that the GDF-1 probe detected an mRNA species in adult brain, adrenal gland, ovary, and oviduct. (See page 23 and Figure 7.) The specification does not identify any tumor (brain or otherwise) associated with GDF-1 nor enable any such diagnostic or therapeutic uses.

With respect to the rejection under 35 USC 112, 1st paragraph, for written description, appellant appears to argue that claim 3, 11-15, 22, and 24-30 do not encompass a gene or genomic sequence. Appellant appears to believe that the claim is limited to a cDNA sequence. This is not agreed with. Language reciting "DNA segment encoding" the named amino acid sequences would still include the genomic sequence.

The arguments concerning degeneracy of the genetic code are not germane as this was not the basis of the rejection.

It is noted that appellant acknowledges that Rankin et al. in Figure 1h describes the genomic structure of murine GDF-1 and that it does in fact contain intronic sequences. This structure is not described in the instant specification.

Appellant is reminded that the Synopsis of Application of Written Description Guidelines (appellant's Exhibit F) are just that, guidelines. They do not supersede the statutes nor the pertinent case law. In addition, the fact pattern here differs from those set forth in the examples.

Unlike Example 9 pointed to by appellant, none of the claims on appeal recites that the encoded protein must have the same activity as the particular human or mouse GDF-1 disclosed. The claims require that the DNA segment encode a mammalian GDF-1 protein with no limitation as to activity.

Appellant's arguments with respect to Figure 5 are not persuasive. The claims are not limited to the particular bands (prominent or otherwise) in the Figure. Appellant argues that these sequences could be readily cloned and sequenced. This rejection is with respect to written description and conveying to one of ordinary skill in the art what the sequences encompassed by the claim are in terms of structure and/or function and not with respect to enablement. As such, this argument is not germane. Likewise, arguments with respect to cloning techniques such as the Sambrook Molecular Cloning manual speak to enablement and not written description.

Appellant argues that claims 24-30 are directed to the specific GDF-1 nucleic acid sequences explicitly disclosed in the specification. This is not agreed with. Again, claim 24 recites "comprising a nucleotide sequence as defined in **an** open reading frame" (emphasis added). This does not require the full open reading frame for GDF-1 but includes subsequences as small as a single nucleotide. The claim does not recite that the open reading frame be for GDF-1. The fact pattern and claim language do not correspond to Example 8 of the Synopsis of Application of Written Description Guidelines relied upon by Appellant.

For the above reasons, it is believed that the rejections should be sustained.

Application/Control Number: 08/966,233
Art Unit: 1631

Page 27

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Mini-Review

Bone Morphogenetic Proteins and Their Receptors: Potential Functions in the Brain

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Transforming growth factors- β (TGF- β s), activins, and bone morphogenetic proteins (BMPs) comprise an evolutionarily well-conserved group of proteins controlling a number of cell differentiation, cell growth, and morphogenetic processes during development. The superfamily of TGF β -related genes include over 25 members in mammals several of which are expressed in the growing nervous system and serve important functions in regionalizing the early CNS. Cultured nerve cells show different responses to these factors. Recent developments have revealed that TGF β s, activins, and BMPs selectively signal to the responding cells via different hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors. The adult brain exhibits specific expression patterns of some of these receptors suggesting neuronal functions not only during development but also in the mature brain. In particular, the brain is expressing high levels of bone morphogenetic protein receptor type II (BMPR-II), activin receptor type I (ActR-I), and activin receptor type IIA (ActR-II). This indicates that osteogenic protein-1 (OP-1/BMP-7), BMP-2, and BMP-4 as well as activins may serve functions for brain neurons. Expression of the receptors partially overlaps in populations of neurons and has been shown to be regulated by brain lesions. This suggests that brain neurons may use receptors BMPR-II and ActR-I to sense the presence of BMPs. This may form a system parallel to the neurotrophin Trk tyrosine kinase receptors regulating neuroplasticity and brain repair. The presence of BMPs in brain is not well studied, but preliminary in situ data indicate that the BMP relatives growth/differentiation factor (GDF)-1 and GDF-10 are distinctly but differentially expressed at high levels in neurons expressing BMPR-II and ActR-I. The receptors mediating responses to these two GDFs remain, however, to be defined. Finally, recent data show that the signal from the activated type I serine/threonine kinase receptor is directly transduced to the nucleus by Smad proteins that become incorporated into transcriptional complexes.

Preliminary in situ hybridization observations demonstrate the existence of different Smad mRNAs. It is concluded that BMPs and their signaling systems may comprise a novel pathway for control of neural activity and offer means for pharmacological interventions rescuing brain neurons. *J. Neurosci. Res.* 51:139-146, 1998. © 1998 Wiley-Liss, Inc.

Key words: BMPs; growth/differentiation factors; serine/threonine kinase receptors; Smad proteins; neuron functions

INTRODUCTION

The superfamily of transforming growth factors- β (TGF β) consists of over 25 members of secreted signaling proteins present in vertebrates as well as invertebrates (review by Kingsley, 1994). These factors have a broad range of biological effects and control processes during development and tissue repair such as cell growth and growth inhibition, cell differentiation, establishment of the dorso-ventral body axis in the embryo, epithelial-mesenchymal remodeling, apoptosis, and secretion of extracellular matrix components (review by Hogan, 1996). The proteins are synthesized as large precursors with the mature protein present in the carboxy-terminal part being released upon proteolytic cleavage. The members of this large superfamily belong to several different subfamilies, e.g., activins and bone morphogenetic proteins (BMPs). Sequence comparisons have made it possible to construct phylogenograms showing the likely evolution of the many members from common ancestral genes (Fig. 1). A shared structural motif among all members of the superfamily is

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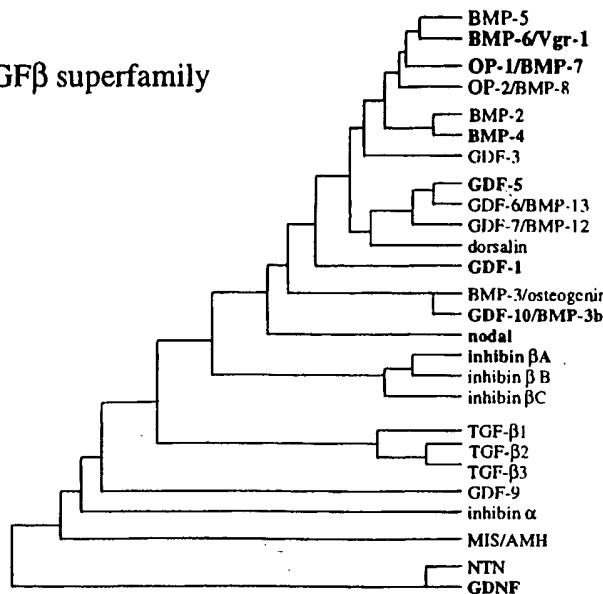
TGF β superfamily

Fig. 1. Schematic dendrogram showing the relationship among vertebrate members of the transforming growth factor- β (TGF β) superfamily. This family includes bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs), osteogenic proteins (OPs), and the Müllerian inhibiting substance/anti-Müllerian hormone (MIS/AMH). These members of the TGF- β superfamily use, as far as known, hetero-oligomeric complexes of type I and type II transmembrane serine/threonine kinase receptors for signal transduction. The distant members glial cell line-derived neurotrophic factor (GDNF) and its close relative neuritin (NTN) bind to specific α -receptor proteins (GDNFR- α and NTNR- α , respectively) and signal via the transmembrane tyrosine kinase receptor Ret. Based on Kingsley (1994), Griffith et al. (1996), Hogan (1996), and Massagué (1996). Factors in bold have been examined in the adult rat brain by the present authors using *in situ* hybridization.

a cystine-knot (Kingsley, 1994; Griffith et al., 1996) forming a rigid structure at the base of the mature protein, a feature also found in, e.g., the neurotrophin family (review by Ebendal, 1992). Accumulating data suggest that members of the TGF β superfamily may have important regulatory roles in the nervous system (review by Kriegstein et al., 1995a). Lately the BMP family within the TGF β superfamily has come into focus for putative functions in the nervous system (recent review by Mehler et al., 1997). Considering the conserved nature of the BMP signaling system, it is interesting to note that the *Drosophila* homologue of vertebrate BMP, *decapentaplegic* (*dpp*), is important in organizing the visual centers of the brain of the fruit fly (Kaphingst and Kunes, 1994).

The exact expression patterns and concentration levels of BMPs in the adult nervous system are known only to a limited extent. This is hampering the understanding of potential functions for these factors in the brain.

However, OP-1 (also known as BMP-7) has been shown to be expressed in the brain of the 2-week-old mouse, but no cellular localization has been given (Özkaynak et al., 1991). In contrast, BMP-6 has been shown to be expressed in neurons of the adult rat brain hippocampus and neocortex (Tomizawa et al., 1995). These authors noted a strong downregulation in expression from the neonatal to the adult brain. At earlier developmental stages there is more evidence for the expression of BMPs in the brain. Furuta et al. (1997) compared the expression patterns of BMP-2, -4, -5, -6 and OP-1 by *in situ* hybridization of the embryonic mouse forebrain and found distinct temporal and spatial overlap among these different BMPs. Activin β A is expressed in the developing cortex and at low levels in the adult rat brain in layers II/III and V/VI of neocortex and CA1 of hippocampus, as well as in the dentate gyrus (Andreasson and Worley, 1995). Moreover, induced excitation results in rapidly raised levels of the activin mRNA in the dentate gyrus (Andreasson and Worley, 1995; Lai et al., 1996). The neonatal rat brain expresses distinctly localized mRNAs for TGF β 2 (frontal and entorhinal and piriform cortex, amygdala, and hippocampus) and TGF β 3 (olfactory bulb, low levels in frontal and piriform cortex; Poulsen et al., 1994), suggestive of developmental actions in these areas. In the adult brain, TGF- β 1 mRNA in the hippocampus has been shown to be upregulated in response to an ischemic lesion, whereas TGF β 2 and - β 3 mRNAs were concomitantly downregulated (Knuckey et al., 1996). High expression levels in the adult brain have also been reported for the BMP-related growth/differentiation factor (GDF)-1 (Lee, 1991) as well as for GDF-10/BMP-3b (Cunningham et al., 1995; Takao et al., 1996). The present authors have used an *in situ* approach to localize these two factors in the brain and have found strong and specific hybridization signals over the hippocampal formation including the dentate gyrus, patterns indicating possible interaction with the receptors for BMPs (Fig. 2) as discussed below.

FUNCTIONS FOR TGF β s IN THE DEVELOPING NERVOUS SYSTEM

A series of effects on neural development by the members of the TGF β superfamily has been described. Well-documented mechanisms involve patterning of the early nervous system. Indeed, BMPs are involved in establishing dorsal and ventral cell fates in the embryo mesoderm even before formation of the nervous system (reviewed by Hogan, 1996; Graff, 1997). BMP-4 and OP-1 are both expressed in the epidermal ectoderm flanking the neural plate (Liem et al., 1995; see also review by Tanabe and Jessell, 1996). BMP-4 is furthermore expressed in the dorsal part of the forming neural tube, and both BMP-4 and OP-1 drive differentiation of

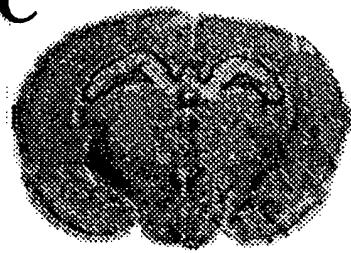
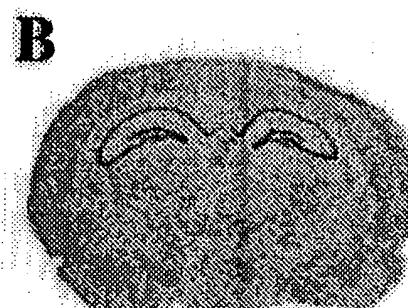
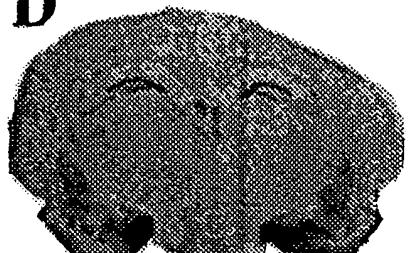
BMPR-II**C****GDF-1****ActR-I****D****ActR-II**

Fig. 2. In situ hybridization patterns in the adult rat brain for serine/threonine kinase receptor mRNAs and one ligand in the BMP/GDF group of growth factors. Coronal sections at the hippocampal level are shown. X-ray films from ^{35}S -labeled oligonucleotide probes are shown (cf. Bengtsson et al., 1995; Söderström et al., 1996; Lewén et al., 1997). **A:** Expression of the bone morphogenic protein receptor type II (BMPR-II). **B:**

Expression of the activin type IA receptor (ActR-I) known to hetero-oligomerize with BMPR-II as well as with activin receptors type IIA (ActR-II) and IIB (ActR-IIB). **C:** Expression of mRNA for growth/differentiation factor 1 (GDF-1). **D:** Expression of mRNA encoding ActR-II. The partial overlap in the expression patterns for the receptors and GDF-1 suggests functional interactions although these remain to be shown.

dorsal cells in the nervous system. Thus, BMPs are important in dorso-ventral specification of cell fates in the early nervous system.

A number of observations mainly from cells in culture suggest additional effects by BMPs and related factors on nerve and glial cells. Neural crest cells appear to be differentially regulated by BMPs. At the hindbrain level, neural crest cells from rhombomeres 3 and 5 are eliminated by apoptosis induced by BMP-4 signaling (Graham et al., 1994). At other levels, BMP-2 seems to instructively drive differentiation to autonomic neurons from multipotent neural crest stem cells (Shah et al., 1996). Also, differentiation into an adrenergic phenotype of trunk neural crest cells is induced by BMP-2 and -4 as well as by OP-1, but not by BMP-6 (Reissmann et al., 1996; Varley and Maxwell, 1996). BMP-2 and to some extent also BMP-6 induce distinct sets of transmitters and neuropeptides in cultured sympathetic neurons (Fann and Patterson, 1994). Moreover, OP-1 has been reported to exert a strong stimulative effect on dendritic growth in

sympathetic neurons (Lein et al., 1995). GDF-5 has been shown to be neurotrophic for embryonic dopaminergic neurons (Kriegstein et al., 1995b) as have TGF β 2 and - β 3 (Poulsen et al., 1994). And not only neurons are affected by the BMPs. BMPs also instructively drive differentiation of astrocytes from subventricular zone progenitor cells (Mehler et al., 1997). Taken together these diverse actions of BMPs on neuronal cells suggest possible involvement in the physiology of neuronal differentiation, induction of transmitter phenotype, and survival and extension of nerve processes, all of which are phenomena involved in neuroplasticity.

BMPs ACT VIA SERINE/THREONINE KINASE RECEPTORS OF TYPE I AND TYPE II EXPRESSED IN THE NERVOUS SYSTEM

The molecular mechanisms for BMP actions have recently been clarified substantially. TGF β s, activins, and BMPs have all been shown to signal via hetero-

Serine/threonine kinase receptors

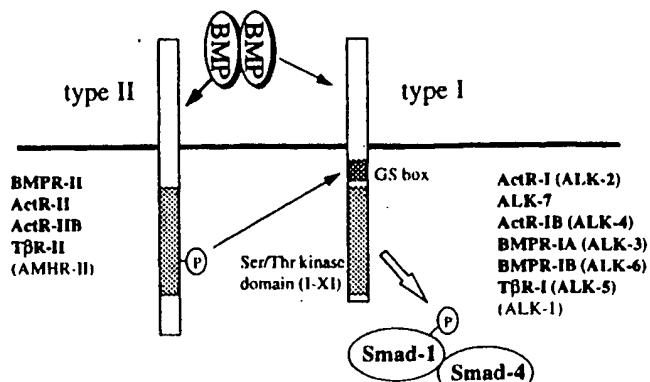


Fig. 3. Cartoon showing the interaction between TGF β superfamily members such as a BMP dimer and the oligoheteromeric complex of transmembrane type I and type II serine/threonine kinase receptors. The kinase domain of the type II receptor, serving as a primary ligand-binding entity, is constitutively phosphorylated and activated. Upon aggregation with the type I receptor, it phosphorylates a juxtamembrane gly-ser-rich motif (GS box) activating the ser/thr kinase domain of the type I receptor. This allows the type I receptor to transduce the signal by phosphorylating a specific receptor-activated Smad protein (see Fig. 4). The four type II receptors and the six type I receptors shown in bold were examined in adult brain tissues by the present authors using *in situ* hybridization. TBR-I and -II, type I and II receptors for TGF β , respectively. ALK, activin receptor-like kinases (type I receptors).

oligomeric complexes of transmembrane serine/threonine kinase type I and type II receptors (reviews by Massagué, 1996; ten Dijke et al., 1996). Seven type I receptor (ALKs, activin receptor-like kinases) genes and five type II genes are known in vertebrates at present (Fig. 3). Several of these are expressed in the developing and adult nervous system (Bengtsson et al., 1995; Söderström et al., 1996). BMPs have a weak affinity for their type I as well as for their type II receptors but require both types of receptors to be present for high-affinity binding and signaling (ten Dijke et al., 1996; Fig. 3). Recent studies have characterized the signaling capacity in these receptors in response to BMP-2, BMP-4, and OP-1. BMPR-II binds these BMPs, but does not signal when activin A is added even in the presence of type I receptors (Liu et al., 1995; Rosenzweig et al., 1995). BMPR-II binds OP-1 and BMP-2 well and forms a signaling complex in the presence of ActR-I or BMPR-IB. BMP-4 works less well than OP-1 in these combinations. BMPR-IA is less effective in signaling with these BMPs when combined with BMPR-II, whereas ActR-IB will not signal at all under these conditions (Rosenzweig et al., 1995). The ability of BMP to signal via BMPR-II and ActR-I in

combination is of obvious relevance for putative brain functions as explained below. It has also been shown that OP-1, in contrast to BMP-4, at higher doses binds to ActR-II and -IIB when ActR-I, BMPR-IA, or -IB are present (Yamashita et al., 1995). This opens further possibilities for combinatorial receptor activation and signaling in brain cells. However, it should be stressed that the receptor preferences for several other BMPs and GDFs of possible relevance for brain function still remain to be characterized.

Only recently has the actual expression of serine/threonine receptors in the adult brain been studied. By *in situ* hybridization, Cameron et al. (1994) localized activin receptors type IIA and IIB (ActR-II and ActR-IIB, respectively) in the rat brain. ActR-II was expressed at higher levels in the brain, and labeling was particularly strong in the dentate gyrus, taenia tecta, amygdala, piriform cortex, entorhinal cortex, and cingulate cortex. Similar findings were made by Bengtsson et al. (1995) who also reported on the expression of activin receptor type IA (ActR-I) in neurons of the hippocampal formation, in particular in CA3 but also in CA1 and dentate gyrus (Fig. 2). Thus, there was only partial overlap in the expression patterns of these two receptors, and we suggested that they may have other interacting partners in some areas of the brain. A third characteristic pattern was found for the bone morphogenetic protein receptor type II (BMPR-II) in a later study of serine/threonine kinase receptors in the developing and adult rat brain (Söderström et al., 1996). BMPR-II was expressed in neurons throughout the hippocampus, habenula, piriform cortex, neocortex, thalamic nuclei, substantia nigra, and cerebellum. In contrast, BMPR-IA and -IB were below detection levels in the adult brain, and ActR-IB was expressed only weakly, but nevertheless in a pattern resembling that of ActR-I (Söderström et al., 1996). The overall result from this study is that ActR-I is the dominating serine/threonine kinase type I receptor in the brain, whereas the dominating type II receptors are BMPR-II and ActR-II (Fig. 2). Thus, the major type I receptor in brain, ActR-I, has two different abundantly expressed type II receptors, BMPR-II and ActR-II, available for interaction as described in the previous section. Although the patterns of expression are somewhat different, it is likely that many neurons coexpress several or all of these receptors. The physiological ligands in the brain for these receptors remain to be defined. Functions for the receptors in brain trauma are nevertheless suggested by their selective regulations in response to injury. Thus, a marked upregulation of ActR-I mRNA was found in the dentate gyrus 6 hr after a mild cortical contusion (Lewén et al., 1997). A similar but less intense increase was found also for the BMPR-II, whereas ActR-II mRNA in contrast was downregulated in the granule cells. The effects were seen only

ipsilateral to the lesion. These observations suggest that BMP receptors are regulated in response to excitation of the brain, possibly in a manner making it feasible to support lesioned neurons with administered BMP factors.

Finally, a novel type I serine/threonine kinase receptor named ALK-7 and preferentially expressed in brain was recently cloned by several laboratories (Tsuchida et al., 1996; Rydén et al., 1996; Lorentzon et al., 1996). This receptor is expressed at very modest levels in the cerebral cortex, hippocampus, thalamic and hypothalamic nuclei, striatum, substantia nigra, and cerebellum. However, ALK-7 expression is strongly upregulated in the dentate gyrus in response to activation of neural activity (Lorentzon et al., 1996), thereby resembling the ActR-I and BMPR-II upregulation in these neurons (Lewén et al., 1997). There is some evidence for a possible interaction between ActR-II and ALK-7 in the presence of activin (Rydén et al., 1996). However, the physiologically relevant type II receptor interacting with ALK-7 and the ligand for ALK-7 remain to be established. It is nevertheless worth noting that there is an interesting overlap in expression of ALK-7 and the putative ligand GDF-10 in the adult dentate gyrus and in the developing cerebellum (unpublished data).

SERINE/THREONINE KINASE RECEPTORS TRANSMIT THEIR SIGNAL VIA CYTOPLASMIC Smad PROTEINS

A remarkable breakthrough in the understanding of TGF β , activin, and BMP signal transduction has been seen during the last year. Thus, it has been shown that the small group of cytoplasmic Smad proteins (first found as *mothers against decapentaplegic*, Mad, in *Drosophila* and *sma-1*, -2, and -3 in *Caenorhabditis*) form a crucial and direct link between the activated membrane receptor and the target genes in the nucleus of the responding cell (review by Massagué et al., 1997). The vertebrates have at least seven different genes encoding Smad proteins, each with a unique function (Fig. 4; Hayashi et al., 1997). Several of the Smads are receptor activated, and as a consequence of this become associated with the shared Smad-4 protein and translocated to the nucleus for incorporation into transcriptional complexes (Lagna et al., 1996; Chen et al., 1996; Zhang et al., 1997b). Most important, there is a direct phosphorylation of Smad-1 by the BMP type I receptors BMPR-IA and BMPR-IB (Hoodless et al., 1996; Kretzschmar et al., 1997). Both these kinases require the simultaneous presence of either BMPR-II or ActR-IIB type II receptors in order to phosphorylate Smad-1 in response to BMP-2. Also Smad-5 is specifically associated with BMP signaling (Suzuki et al., 1997). In contrast, Smad-2 and -3 mediate signals evoked by activin (via ActR-IB) and TGF- β (via T β R-I;

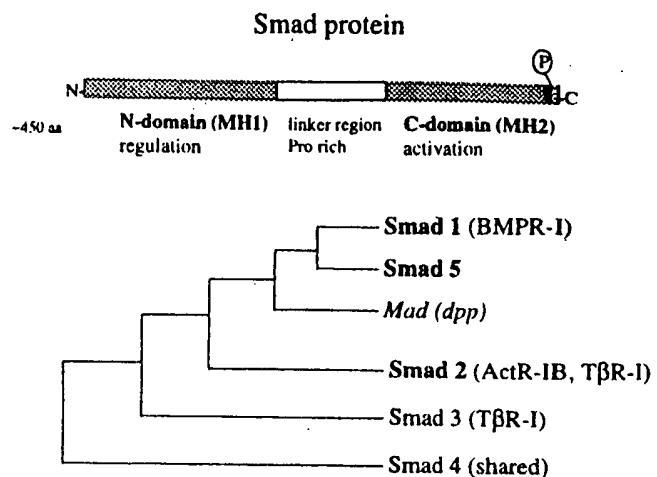


Fig. 4. Schematic structure of the Smad proteins, cytoplasmic messenger molecules linking the activated type I receptor to transcriptional activation complexes (upper panel). The amino and carboxy termini of the Smad proteins are linked by a proline-rich domain. The ligand-activated Smad proteins are phosphorylated in their C termini on residues -SSXS- rendering the protein active (Kretzschmar et al., 1997). The lower panel shows the relationship among the vertebrate Smads and their *Drosophila* Mad protein (encoded by the gene *mothers against decapentaplegic*). Smad-1, -2, -3, and -5 are receptor activated and combine with Smad-4 after phosphorylation by the involved type I receptor. Smad-1, -2, and -5 expression is currently under study in the brain by *in situ* hybridization.

review by Massagué et al., 1997). In contrast to the established activation of Smad-1 by BMPR-IA and BMPR-IB after stimulation by BMP-2, little is known about the signaling from ActR-I (Massagué et al., 1997), the type I receptor most abundantly expressed in adult brain neurons (Fig. 2; Söderström et al., 1996). Thus, none of the known Smad proteins are activated by ActR-I. We have examined the expression of Smad-1, -2, and -5 in the adult rat and mouse brain by *in situ* hybridization and find weak expression in hippocampal neurons of all these genes (our unpublished data). The possibility that other Smad proteins than those presently examined mediate signaling in response to activation of ActR-I, and thus are likely to be of great importance in brain tissue, needs further investigation.

Very recently the structure of the Smad-4 carboxy terminus has been solved. It was shown to comprise a trimeric structure forming a disk with a flat surface that binds to the receptor-activated Smads (-1, -2, -3, and -5) after phosphorylation of serine residues in their carboxy termini (review by Wrana and Pawson, 1997). An interesting issue is the nature of the transcription factors with which this Smad heterohexameric complex becomes associated to control transcriptional activation. One such candidate transcription factor, which binds to an activin-

responsive DNA element and interacts with the Smad-2 protein, was recently isolated (Chen et al., 1996). This transcription factor, designated FAST-1, is a novel member of the HNF-3/fork head family of winged-helix transcription factors that also includes the brain-specific transcription factor BF-1 (Tao and Lai, 1992). Very little is yet known about the presence and regulation of these and related transcription factors that possibly serve functions in the BMP signaling pathway in the brain. Interestingly, Furuta et al. (1997) recently found that BF-1 expression is downregulated in the embryonic lateral telencephalon in response to ectopic application of BMP-4. Since the winged-helix transcription factors are encoded by at least seven genes, several of which have been reported to be expressed in the early brain, there is a need for further examination of their relevance for BMP signaling in the adult CNS. It should also be noted that Mad proteins in some cases may activate transcription on their own, by sequence specific binding via their amino-terminal domain (Kim et al., 1997).

Of obvious interest is the definition of the set of downstream genes being regulated by the activated Smad-containing transcriptional complexes. At least during development these genes are likely to encode various transcription factors determining the identity of the immature cell. Indeed, BMP-4 has been shown to induce the expression of the homeobox genes *Msx1* and *Msx2* (review by Davidson, 1995) in the early mouse telencephalon (Furuta et al., 1997) and in rhombomeres 3 and 5 of the chicken embryo (Graham et al., 1994), respectively. It seems fair to say that the genes being controlled by BMPs in the adult brain are urgently in need of study.

GENETIC ANALYSIS OF BMP FUNCTION IN THE CNS

It is clear from the overall expression pattern of serine/threonine kinase receptors in the adult brain that BMP signaling pathways may serve important functions for neuroplasticity and synapse functions. Similar functions have been implicated also for neurotrophins and their Trk tyrosine kinase receptors (see review by Schuman, 1997). The serine/threonine kinase membrane receptors may constitute an evolutionarily conserved system for control of development of and plasticity in nerve nets. Thus, it may be of significance that the addition of TGF β to isolated nerve tissue from *Aplysia* induces long-term synapse facilitation of synapses between sensory and motor neurons (Zhang et al., 1997a). However, several circumstances prevent a clear view on the BMP functions in brain plasticity. One is the apparent redundancy among potential ligands activating the BMP receptors. During brain development this overlap in the expression of several BMPs allowing for compensatory

actions have been used to explain the mild phenotypic manifestations of eliminating the gene encoding any single BMP factor (Furuta et al., 1997). Another fact is that elimination of the BMP genes of interest in several cases are embryo lethals and that studies on brain development consequently are restricted to early stages (reviewed by Hogan, 1996).

It is thus a challenge to apply nerve-tissue specific or inducible gene knock-outs to further define the function of different BMPs in the adult mouse brain. Such an approach is feasible using the Cre-loxP system as recently shown by Tsien et al. (1996). Consequently, the application of such techniques will delineate the function of each BMP or combinations of BMPs in the brain. Moreover, using such a genetic approach to dissect neuroplasticity may also open for analysis the possible interactions between the serine/threonine kinase receptor pathway controlled by BMPs and related TGF β superfamily members with the tyrosine kinase receptor pathway activated by neurotrophins. Besides increasing basic understanding of brain functions, the outcome may result in novel approaches for pharmacological intervention to rescue degenerating neurons.

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Rapid Communication

Glial Cell Line-Derived Neurotrophic Factor Stimulates Fiber Formation and Survival in Cultured Neurons From Peripheral Autonomic Ganglia

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Human recombinant glial cell line-derived neurotrophic factor (GDNF) was tested for its ability to stimulate fiber formation and neuron survival in primary cultures of peripheral ganglia dissected from the chicken embryo. GDNF, first characterized by its actions on central nervous system (CNS) neurons, had a marked stimulatory effect on fiber outgrowth in sympathetic and ciliary ganglia. Weaker responses were evoked in sensory spinal and nodose ganglia and in the ganglion of Remak. In addition, survival of neurons from the sympathetic and ciliary ganglia was stimulated by GDNF at 50 ng/ml. The effects were not mimicked by the distant but related protein transforming growth factor beta 1 (TGF β 1). The profile of neurons stimulated by GDNF is also distinct from the patterns of stimulation shown by nerve growth factor (NGF), stimulating strongly sympathetic but not ciliary ganglia, and ciliary neurotrophic factor (CNTF), stimulating mainly the ciliary ganglion. Moreover, using *in situ* hybridization histochemistry, GDNF was demonstrated to be present in the pineal gland in the newborn rat, a target organ for sympathetic innervation. The present results suggest that GDNF is likely to act upon receptors present in several autonomic and sensory neuronal populations. GDNF may serve to support fiber outgrowth and cell survival in peripheral ganglia, adding yet one more trophic factor to the list of specific proteins controlling development and maintenance of the peripheral nervous system.

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Key words: trophic factor, TGF β , chicken embryo, tissue culture, bioassay

INTRODUCTION

Development, selective survival, and function of neurons are regulated by cellular interactions mediated by a host of neurotrophic molecules. Thus, proteins with a well-characterized ability to support neurons include the family of neurotrophins [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins-3 and -4 (NT-3 and NT-4)], ciliary neurotrophic factor (CNTF), acidic and basic fibroblast growth factors (aFGF and bFGF, respectively), and insulin-like growth factors (IGF-1 and IGF-2).

A recent addition to the list of proteins with trophic effects on neurons is glial cell line-derived neurotrophic factor (GDNF) (Lin et al., 1993). GDNF was initially documented to support survival, differentiation, and high-affinity dopamine uptake in fetal dopamine neurons from the ventral mesencephalon *in vitro*. The active molecule was purified and partially sequenced and subsequently DNA clones encoding the novel neurotrophic factor were isolated from rat cDNA and human genomic DNA (Lin et al., 1993). Analysis of the sequences obtained showed that GDNF, a 134-amino-acid protein in its mature form, is a member of the transforming growth factor beta (TGF β) superfamily of growth and transforming factors (see review by Massagué, 1990) but is not closely related to any of the many previously known

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members of this family (Burt, 1992; Lin et al., 1993; Burt and Law, 1994). When expressed as a recombinant protein produced in bacteria, the GDNF, after proper refolding (Lin et al., 1993) had the expected dopaminotrophic activity originally described in the glial cell line from which it was derived (Schubert et al., 1974). The TGF β proteins, the neurotrophins, and the platelet-derived growth factors A and B (PDGFs) all share some protomeric structural motifs rendering them members of the cystine-knot superfamily of growth factors (McDonald and Hendrickson, 1993). GDNF, like the other cystine-knot proteins, form homo- or heterodimers that are the biologically active entities. It is thus interesting that neurotrophic activities have been ascribed not only to NGF and the other neurotrophins, but also to PDGF and to TGF β s. Very recently, the structural characterization of human chorionic gonadotropin (hCG) has shown that it is also a member of the cysteine-knot family of proteins (Lapthorn et al., 1994) but the neurotrophic activity of hCG has not yet been reported.

So far, studies of the effects of GDNF have involved neurons of the central nervous system (CNS) such as the mesencephalic dopamine neurons and α -motoneurons (Lin et al., 1993). In addition, GDNF is expressed in the rat striatum and in other areas of the brain during development (Schaar et al., 1993; Strömberg et al., 1993). Moreover, in the adult rat brain, kainate-induced epileptic seizures induce GDNF expression in the granule cells of the adult dentate gyrus (Humpel et al., 1994), and pilocarpine-induced seizures lead to upregulation of GDNF in the striatum (Schmidt-Kastner et al., 1994).

In the present report we demonstrate that GDNF also has the ability to stimulate peripheral neurons. We document neurotrophic effects of GDNF in a series of sympathetic and parasympathetic autonomic as well as some sensory peripheral neurons in culture. It is shown that GDNF has substantial trophic effects, particularly on some autonomic neuron populations, and that these patterns of stimulation are distinct from those evoked by the neurotrophins (NGF, BDNF, NT-3, and NT-4) as well as CNTF and TGF β 1.

MATERIALS AND METHODS

Ganglia from chicken embryos at day 9 of incubation were explanted as intact ganglia for a fiber-outgrowth assay (Ebendal et al., 1978; Hedlund and Ebendal, 1978; Ebendal et al., 1980, 1984; Ebendal, 1989) or, in the case of the sympathetic paravertebral trunk ganglia and the ciliary ganglion, dissociated for a neuron-survival assay (Ebendal et al., 1985; Kullander and Ebendal, 1994). Sympathetic paravertebral ganglia and sensory spinal ganglia (dorsal root ganglia) were dissected from the lumbosacral region of the embryo. The

ciliary ganglion was taken from the orbit, the nodose ganglion from the vagus nerve rostral to the heart. Finally, the ganglion of Remak was isolated from the mesorectum of the embryo (Hedlund and Ebendal, 1978). The effects of GDNF in cultures of the sensory trigeminal and spinal (dorsal root) ganglia were also tested. Ganglia or dissociated neurons were placed in collagen gels for culture as detailed by Ebendal (1989). All findings were repeated at least twice in independent experiments yielding the same results. The basic finding of a strong GDNF stimulation of the sympathetic ganglion has been repeated in more than 25 cultures established over a period of more than 6 months in our laboratory. Likewise, the survival data are based on counting of several hundred neurons on several occasions.

Recombinant human GDNF was obtained from Syrogen, Inc. (Boulder, CO). The protein was produced in bacteria, refolded to yield an active molecule, and purified as a non-glycosylated disulfide-bonded homodimer as described (Lin et al., 1993). Human recombinant TGF β 1 was obtained from Boehringer Mannheim (Darmstadt, Germany). Both proteins were aliquoted to avoid repeated freeze-thawing cycles and added to the culture medium at the final concentrations indicated for each experiment. For positive effect on neuron survival, purified mouse β NGF (Ebendal et al., 1984) was added to sympathetic neurons at 5 ng/ml. To support ciliary neuron survival, an extract of the choroid coat including the pigment epithelium from the 18-day-old chicken embryo was added at a final concentration of 200 μ g of total protein/ml of medium (Ebendal, 1987).

Ganglia and dissociated neurons were observed under darkfield or phase contrast optics, fiber outgrowths observed, neurons counted, and microphotographs taken after 2 days in culture.

For *in situ* hybridization, E17 rat fetuses were taken from pregnant Sprague-Dawley rats that had been killed by neck dislocation under deep ether anesthesia. The heads of the fetuses were rapidly frozen on dry ice. Newborn rats were killed by decapitation and the heads frozen on blocks of dry ice. Cryostat sections (14 μ m thick) of the fetal and neonatal brains were cut and placed on coated slides (ProbeOn, Fisher Biotech, Orangeburg, NY). To perform *in situ* hybridization (Dagerlind et al., 1992), sections were thawed and hybridized with two antisense oligonucleotide probes (both 50-mers, positions corresponding to nucleotides 456–505 and 540–589, respectively, in the sequence deposited under GenBank accession number L15305; see Lin et al., 1993). These two oligonucleotide probes had no similarities to sequences deposited in GenBank and generated identical *in situ* hybridization patterns in tissue. As a negative control, a random probe of the same length and GC content was applied to adjacent sections. This

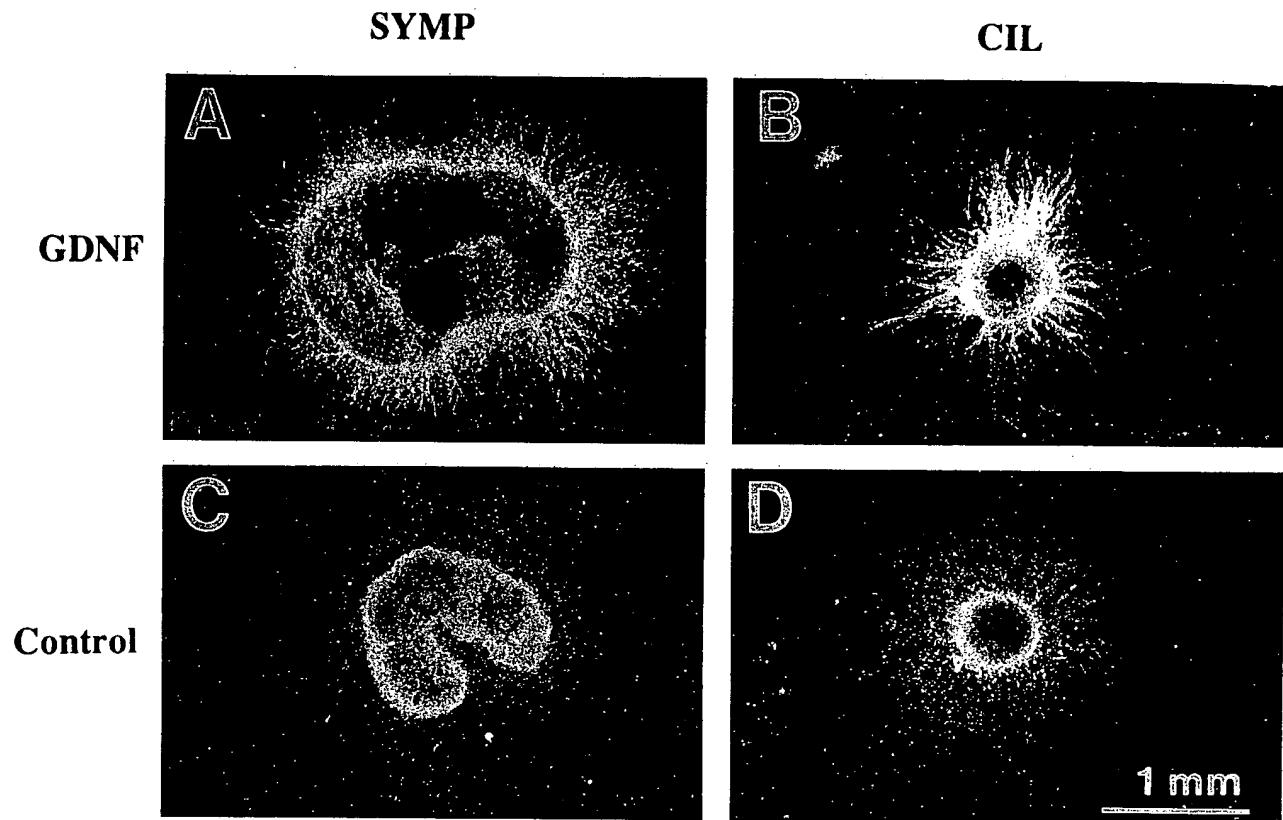


Fig. 1. The response to GDNF in explanted sympathetic and ciliary ganglia. **A:** A sympathetic ganglion cultured for 2 days in the presence of GDNF at 100 ng/ml. A dense, short fiber outgrowth is evident around the ganglion. **B:** The same conditions as in A but with a ciliary ganglion. The formation of

fiber fascicles is evident. **C:** A negative control with a sympathetic ganglion cultured for 2 days without the support of GDNF. **D:** A ciliary ganglion in control medium. Darkfield micrographs of living cultures.

random probe did not give rise to any signals above background in examined tissues. The oligoprobes were end labeled with ^{35}S -dATP using terminal deoxyribonucleotidyl transferase. The probes were then purified (Nensorb columns) and applied to the tissue sections at 42°C overnight in a hybridization solution. Following this, the slides were rinsed in 1× SSC at 54°C, dehydrated in a series of ethanols, and air-dried. The slides were dipped in Kodak NTB-2 film emulsion and exposed for 6–8 weeks at –20°C, developed in a photographic developer, fixed, lightly stained with cresyl violet, and mounted. The sections were observed and photographed with dark- and brightfield illumination (Nikon Microphot FX microscope).

RESULTS

GDNF, present at a concentration of 50–100 ng/ml, was consistently found to evoke marked fiber outgrowth in the explanted sympathetic and ciliary ganglia cultured for 2 days (Fig. 1A,B). The outgrowth consisted

of dense, short fibers around the sympathetic ganglia. From the ciliary ganglion, GDNF evoked the formation of fairly thick fiber fascicles. The concentrations tested were 0.5, 5, 50, 100, 200, 400, and 1,000 ng/ml of GDNF in the medium and optimum fiber responses were obtained in the sympathetic ganglia with 50 and 100 ng/ml. The resulting outgrowth in the sympathetic ganglion was less prominent than that evoked by NGF at 3–5 ng/ml (Ebendal, 1989), but the fibers were dense and markedly tufted. For comparison, representative NGF-induced outgrowth responses in sympathetic ganglia in this assay can be found in a recent report by Kullander and Ebendal (1994).

The ciliary ganglion responded by fiber outgrowth to GDNF in the range of 5–1,000 ng/ml, thus at a wider range of concentrations than found effective to evoke fiber outgrowth in the sympathetic ganglion. Control cultures of sympathetic and ciliary ganglia without added GDNF were totally devoid of fiber outgrowth (Fig. 1C,D).

Some effects of GDNF on fiber outgrowth were

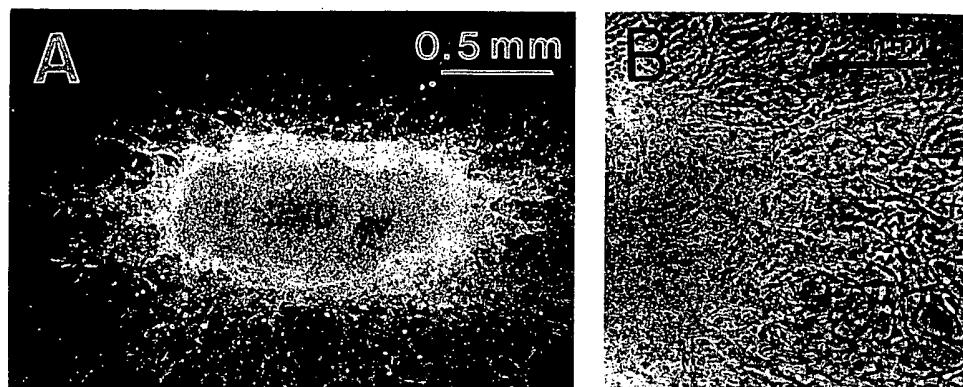


Fig. 2. Effects of GDNF on fiber outgrowth in the nodose ganglion. Some thick fiber fascicles have formed from this sensory ganglion cultured for 2 days with 100 ng/ml of GDNF.

A: Darkfield overview of the ganglionic explant. **B:** Phase contrast close-up showing the heavily fasciculated fibers induced by GDNF.

also observed in the nodose ganglion (Fig. 2A). A few thick fiber fascicles (Fig. 2B) were formed from this sensory ganglion in response to 100 ng/ml of GDNF over the 2 day culture period. It should be stressed that the outgrowth response was markedly weaker than that evoked by NT-3 at 5–10 ng/ml (for a comparison, see the results obtained in this assay presented by Ernfors et al., 1990) in this epidermal placode-derived ganglion. GDNF also weakly stimulated some fiber outgrowth in the spinal dorsal root ganglia (not shown) and in the trigeminal ganglion (data not shown). The effect of GDNF on fiber outgrowth from Remak's ganglion (data not shown) was only marginal at 50 ng/ml, in contrast to the very strong fiber outgrowth evoked in this ganglion by NT-3 at concentrations of 3–5 ng/ml (Ernfors et al., 1990; Kullander and Ebendal, 1994).

The specificity of the GDNF stimulation in the ganglia was tested by comparisons of neurotrophic responses evoked by TGF β 1. Sympathetic ganglia cultured for 2 days with TGF β 1 at 10 and 100 ng/ml were found to lack fiber outgrowth and thus resemble the control explants in ordinary culture medium (Fig. 3A,B), in contrast to the dense fiber outgrowth formed in response to GDNF at 100 ng/ml (Fig. 3C,D). The specificity of the GDNF stimulation was tested also in the ciliary ganglion. In control medium no formation of fibers occurred (Fig. 4A). The ciliary ganglion likewise showed no response to TGF β 1 at 10 or 100 ng/ml (Fig. 4B) present for 2 days in the culture. In contrast, GDNF present at 100 ng/ml resulted in the formation of dense fiber fascicles (Fig. 4C).

In addition to selective stimulation of fiber outgrowth in some peripheral ganglia, GDNF has survival effects on dissociated ganglionic neurons in culture. A dose-response relationship between survival of sympathetic neurons and the presence of GDNF in the concen-

tration range 1–100 ng/ml is shown in Figure 5A. Half-maximum survival effect was seen at about 50 ng/ml GDNF and represents rescue of nearly half of the seeded neurons. Increasing the dose of GDNF above 100 ng/ml did not further enhance survival rate (data not shown). A similar response was found in the dissociated ciliary neurons stimulated by GDNF (Fig. 5B). For sympathetic neurons, NGF at 5 ng/ml will rescue close to 100% of the neurons. The same is true for ciliary neurons when grown with a choroid extract from the eye of the embryonic day 18 chicken (Ebendal, 1987).

In order to examine the presence of GDNF mRNA in target tissue for peripheral autonomic innervation, we used the rat since the chicken GDNF sequence is not yet known. In situ hybridization using two non-overlapping GDNF specific oligonucleotide probes revealed a strong signal in the pineal gland of the newborn rat (Fig. 6A). The signal appeared to be present over most but not all pinealocytes (Fig. 6B). A similar signal was seen also in the pineal gland of the fetal rat at embryonic day 17 (not shown).

DISCUSSION

In the present report we show that GDNF has a pattern of stimulating ganglionic neuron populations distinct from those described earlier for the neurotrophins NGF, BDNF, NT-3, and NT-4, as well as from that of the ciliary neurotrophic factor CNTF. Thus, the Remak ganglion is strongly stimulated by NT-3 (Ernfors et al., 1990; Kullander and Ebendal, 1994) due to its abundant expression of the tyrosine kinase receptor TrkC (Williams et al., 1993), whereas GDNF had very limited stimulatory action on this ganglion. The sympathetic ganglion was markedly stimulated by GDNF but not to the extent of that seen with NGF. The response involved

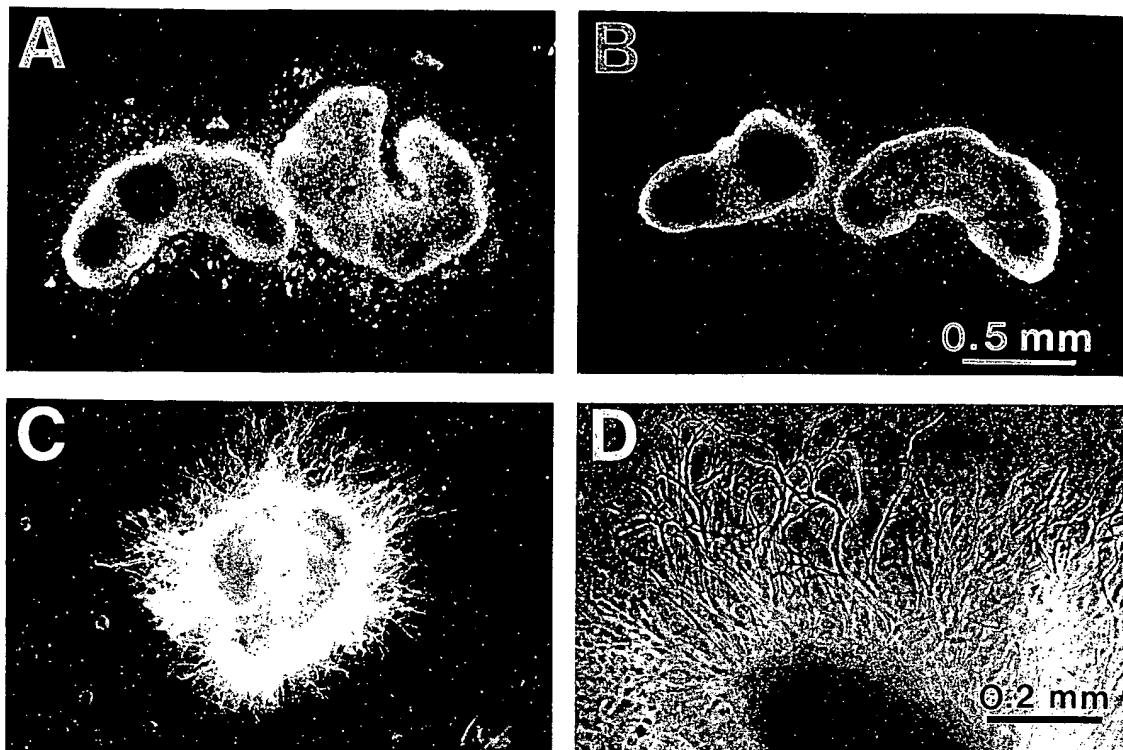


Fig. 3. Specificity of the GDNF stimulation in the sympathetic ganglion. **A:** Sympathetic ganglion in control medium after 2 days of culture. **B:** A sympathetic ganglion cultured for 2 days with TGF β 1 at 100 ng/ml. **C:** Sympathetic ganglion with GDNF at 100 ng/ml present in the medium for the culture

period of 2 days. Only with GDNF present are fibers formed. Darkfield microscopy. **D:** Phase contrast detail of fiber outgrowth from a sympathetic ganglion grown for 2 days with GDNF at 100 ng/ml.

both a survival and a fiber outgrowth response. On the other hand, GDNF also stimulated the ciliary ganglion which NGF does not. It remains to be examined whether there are time-dependent switches in the responsiveness to GDNF in the autonomic and sensory neurons examined here in analogy to changing dependency for different neurotrophins found earlier in sensory neurons (Buchmann and Davies, 1993). This could provide insight into potential temporal windows during development when these peripheral neurons depend on GDNF. *In situ* hybridization has been utilized to study GDNF expression and its relationship to innervation. It has been shown that there is an early expression of GDNF in the brain which is later downregulated (Schaar et al., 1993; Strömberg et al., 1993), and such an expression pattern may argue for developmental regulation of GDNF to serve neurosupportive functions during restricted periods of neurodevelopment.

Embryonic tissue extracts and explants have, in a number of cases, been shown to stimulate ganglionic neurons in culture (Ebendal et al., 1980, 1984, 1985; Ebendal, 1987). Many of these effects have been as-

cribed to the presence of CNTF, but also to aFGF and bFGF. GDNF is one more factor which may have contributed to these observed stimulatory effects.

In order to examine if GDNF mRNA is present in any target areas for peripheral innervation we used the perinatal rat, since the GDNF gene has not yet been sequenced from the chicken. In view of the marked effects of GDNF on the chicken sympathetic ganglia, we chose to study one target organ for the sympathetic superior cervical ganglion, i.e., the pineal gland. Consistent with the *in vitro* data, the pineal gland was found to express relatively high levels of GDNF mRNA during late prenatal and early postnatal stages of development (Fig. 6). Thus, GDNF may be one of the target-derived trophic factors stimulating the sympathetic nerves that innervate targets such as the pineal gland.

In the present study, specific activities of GDNF on the responsive peripheral neurons were found in the range of 50–100 ng/ml of the factor. This contrasts with the lower concentrations found to evoke tyrosine hydroxylase (TH) induction in cultured ventral mesencephalic neurons. Thus, Lin et al. (1993) found GDNF to be

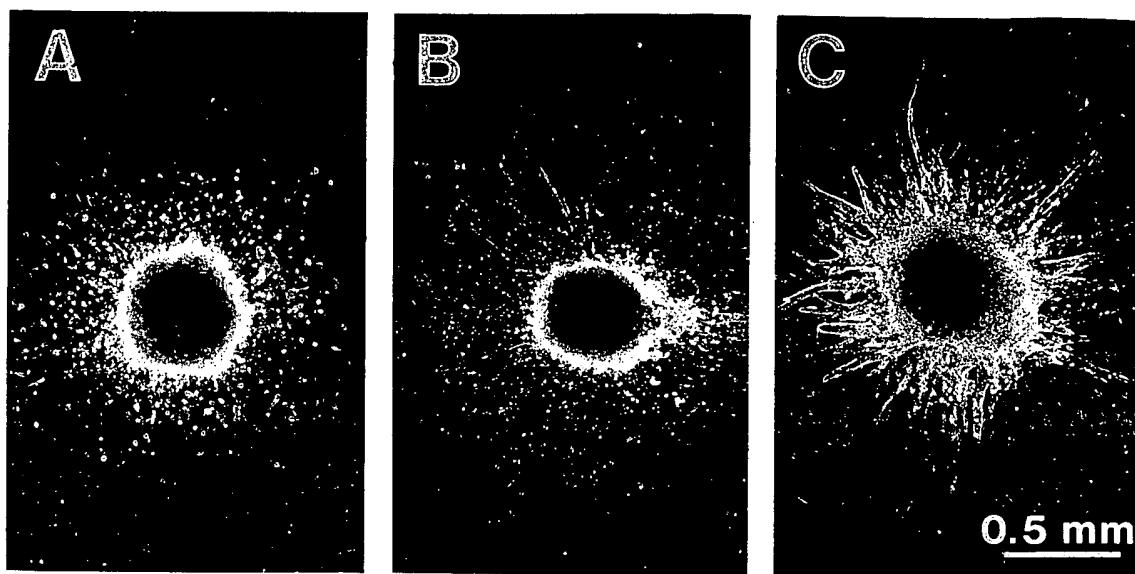


Fig. 4. Specificity of the GDNF stimulation in the ciliary ganglion. **A:** Ganglion grown in control medium for 2 days. No formation of fibers occurs. **B:** A ciliary ganglion showing no response to TGF β 1 at 100 ng/ml. **C:** A ciliary ganglion cul-

tured for 2 days with GDNF present at 100 ng/ml. GDNF, but not TGF β 1, elicited dense fiber fascicles. Darkfield microscopy.

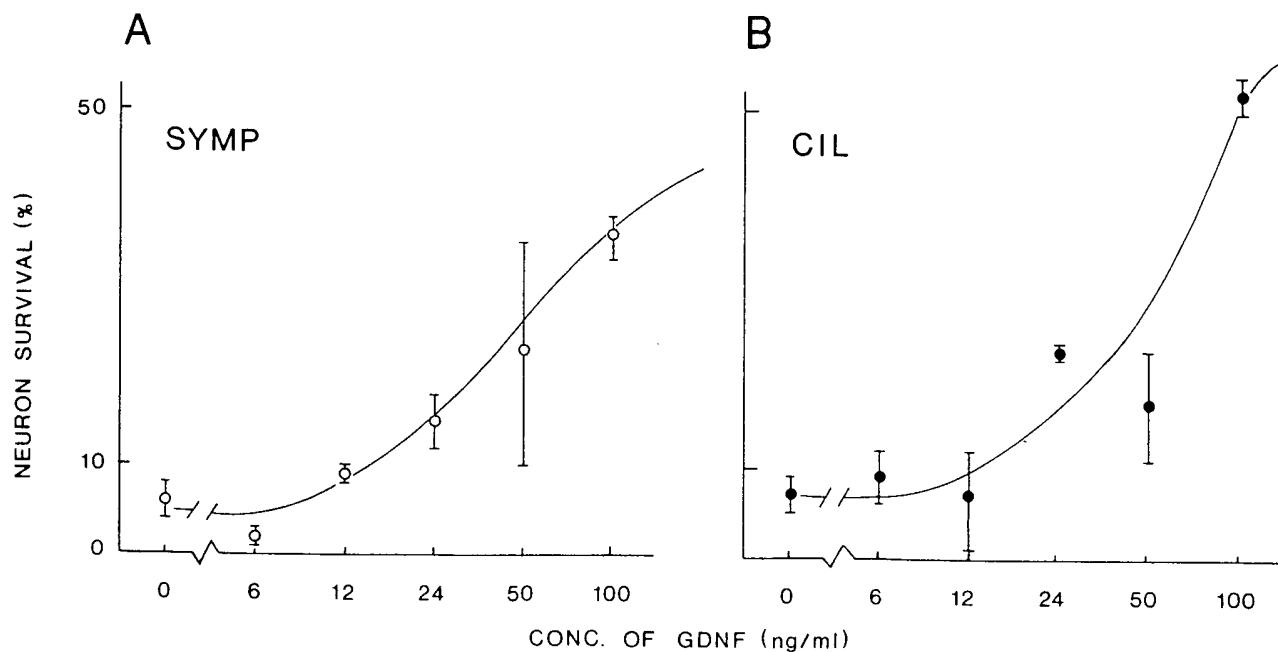


Fig. 5. Survival effect of GDNF on dissociated ganglionic neurons in culture. **A:** Dose-response curve for survival of sympathetic neurons in response to GDNF. **B:** The same experiment shown for ciliary ganglionic neurons. Each graph is

based on two independent experiments, with each value based on observations of the survival in several hundred neurons. Mean value and range are shown. Survival is shown relative to the number of initially seeded neurons.

effective at a concentration of 0.04–1 ng/ml, which is in line with what is generally expected for a high-affinity interaction between a growth factor and its specific receptor. It is therefore possible that the present effects are

the result of GDNF mimicking a related endogenous growth factor that might act on the peripheral neurons at higher efficiency.

The present experiments showed that GDNF stim-

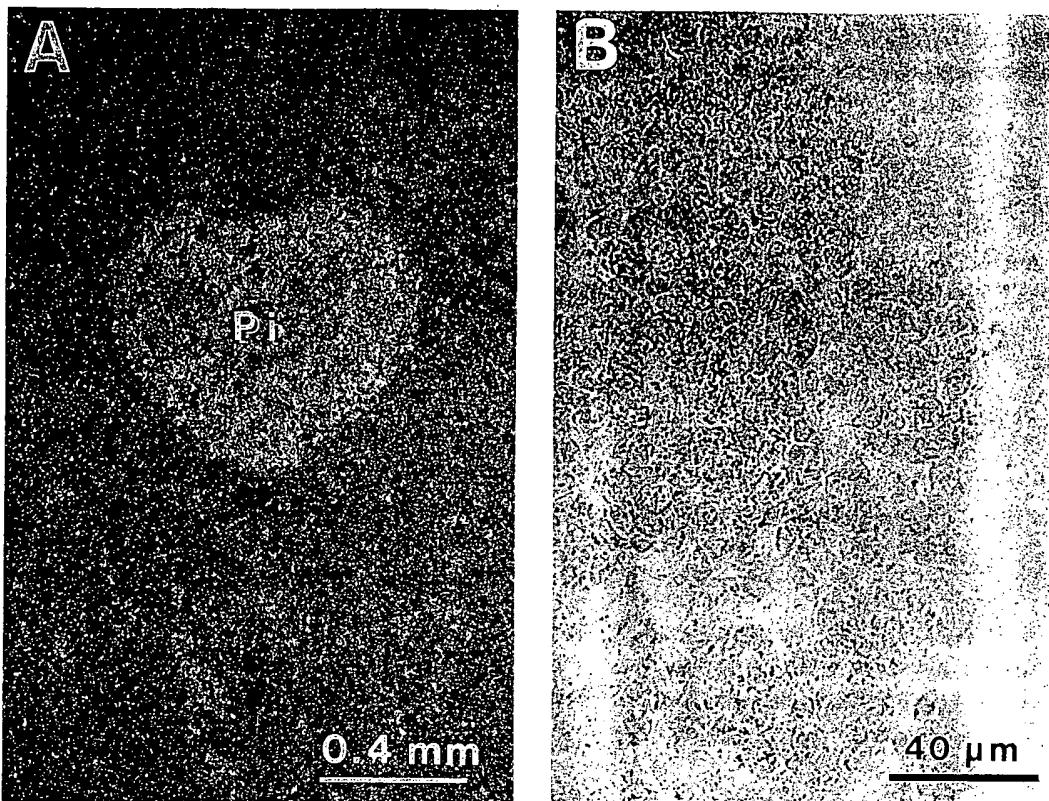


Fig. 6. *In situ* hybridization with an oligonucleotide probe complementary to rat GDNF mRNA. **A:** Darkfield overview of the pineal gland (Pi) of the newborn rat. **B:** Brightfield micrograph showing hybridization signal over many but not all pinealocytes.

ulated the ciliary ganglion to form nerve fibers in a concentration range of 5–1,000 ng/ml, thus at a wider range of concentrations than was effective for the sympathetic ganglion. This may reflect differences in the growth properties of the ganglia when taken to culture and not necessarily point to different receptor mechanisms. We have earlier found that NT-3 marginally stimulates fiber formation in the ciliary ganglion (Ernfors et al., 1990). The response to GDNF is stronger than that elicited by NT-3 and also appears stronger than the fiber outgrowth response evoked by recombinant CNTF in explanted ciliary ganglia (see Carri et al., 1994).

Before the cloning of GDNF, members of the TGF β superfamily of growth factors have been considered to possess various neurotrophic and neuron differentiating properties. Thus TGF β 1 promotes the survival of motoneurons in culture, but fails to support the survival of dissociated sympathetic neurons (Martinou et al., 1990), in contrast to the action of GDNF shown here. Activin has been found to stimulate survival in some populations of neurons including the chicken embryo retina (Schubert et al., 1990), but has failed to support the survival of dissociated ciliary ganglionic neurons in an assay similar to the one used here. Again, this

contrasts with the supportive effect of GDNF on dissociated ciliary ganglion neurons in the present report.

TGF β 1, -2, and -3 have all been localized in the nervous system using specific peptide antibodies (Flanders et al., 1991). TGF β 1 was mainly found in the meninges, whereas TGF β 2 and TGF β 3 were found in neurons and radial glia. It was also found that TGF β 2 and -3 inhibited the survival of ciliary ganglion neurons in the presence of an eye extract normally supporting these neurons in dissociated cultures (Flanders et al., 1991). This could again indicate the presence of receptors for TGF β s being present on these primary neurons. Supportive of this, TGF β 1 and -2 were found to enhance sensory dorsal root ganglion neuron survival and result in increased levels of substance P (Chalazonitis et al., 1992), actions suggested to be exerted synergistically with NGF. Other additive or synergistic effects of GDNF with the neurotrophins are thus possible, but have yet to be studied.

The present data suggest that a receptor mechanism for GDNF or a related trophic factor is present in developing sympathetic and ciliary neurons and that the receptor specificity is such that GDNF is an agonist ligand whereas TGF β 1 fails to elicit the signaling events lead-

ing to fiber outgrowth or neuron survival in these neurons. Several receptors for the members of the TGF β family have been identified (see reviews by Massagué, 1992; Lin and Lodish, 1993), but the GDNF receptor, or receptors, remains uncharacterized. It seems likely that the receptor molecules for GDNF are related to the other signaling receptors mediating the actions of the members of the TGF β superfamily. Activin receptors (Matthews and Vale, 1991; Attisano et al., 1992) and a TGF β receptor serine/threonine kinase receptor have been cloned (Lin et al., 1992) and classified as type II transmembrane receptors of approximately 75 kDa. Also several type I receptors have been cloned (ten Dijke et al., 1994). It is considered that TGF β actions are mediated via signal transduction involving the heterodimerization of receptors of the two types and that type II receptors exhibit a higher degree of specificity for their cognate ligands than the type I receptors (ten Dijke et al., 1994). It has recently been shown that the receptors of class II bind the TGF β and as a second step recruit receptors of type I, which as a result is phosphorylated on serines and threonines as an initial step in a cascade of signaling phosphorylation of downstream proteins (Wrana et al., 1994). Recently, a chicken type II TGF β receptor has been cloned (Barnett et al., 1994) and has considerable sequence similarity to the mammalian TGF β receptor II in the kinase domain but is highly divergent in the N-terminal ligand binding part of the receptor. Whether this receptor is mediating the GDNF effects seen presently in peripheral chicken neurons remains to be studied.

Since TGF β induces the formation of extracellular matrices (Massagué, 1990; Lin and Lodish, 1993) similar effects may be evoked by GDNF. Such a mechanism might be particularly important in the development of the peripheral nervous system where growing axons come into contact with a rich extracellular matrix. It has also been shown that various molecular components of such matrices enhance the formation of fiber outgrowth from the ganglia studied here (Carri et al., 1988). A further possible mechanistic link between GDNF and the extracellular matrix involves the possibility that such matrices bind locally secreted GDNF to enhance nerve growth and neuron differentiation, as has been shown for other members of the TGF β superfamily (Massagué, 1990; Lin and Lodish, 1993).

The importance of GDNF for sympathetic ganglion development *in vivo* remains unclear. Null mutations of members of the neurotrophin family in mice suggest that GDNF does not have a function that is redundant to that of the neurotrophins. Thus the superior cervical ganglion will lose neurons in the mice homozygous for the null mutation. However, the temporal relationships have not been defined so the contribution of GDNF to survival of peripheral neurons cannot yet be established. Homozy-

gous NGF null mutant mice (Crowley et al., 1994) show marked reductions in the sympathetic superior cervical ganglion, with up to 80% reduction of the ganglionic volume, losses of neurons, and the presence of abundant pycnotic nuclei 3 days after birth. Moreover, inactivation of the NT-3 gene in mice (Ernfors et al., 1994; Fariñas et al., 1994) leads to a 50% loss in neurons in the superior cervical ganglion. Thus, the normal expression of GDNF is not sufficient to maintain the sympathetic neurons in animals lacking NGF or NT-3 during development. It is possible that the small remaining population of neurons may represent GDNF-dependent cells or that GDNF affects a wider population of NGF- and NT-3-dependent neurons during other time windows of development (cf. Buchmann and Davies, 1993).

These complexities notwithstanding, we have demonstrated that GDNF has a stimulatory effect on sympathetic and ciliary ganglionic neurons. The data suggest the possibility that GDNF, or an endogenous ligand similar to GDNF, has potent neurotrophic functions supporting cell survival and neurite promotion in these neurons during stages of their development, with a spectrum of responsive neurons not shared by other known trophic factors. The present report also shows that sympathetic ganglion explants offer a robust and simple way to assay for the activity of GDNF, and possibly also for related molecules.

ACKNOWLEDGMENTS

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Potentiating Interactions Between Morphogenetic Protein and Neurotrophic Factors in Developing Neurons

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mRNA for bone morphogenetic protein receptor type II (BMPR-II) was mapped to different neurons in peripheral ganglia and spinal cord of the chicken embryo. The expression of this serine/threonine kinase receptor partially overlaps with that of tyrosine kinase receptors Trk and Ret. Biological activities of osteogenic protein-1 (OP-1), a documented ligand for BMPR-II, were tested in explanted embryonic chicken ganglia and dissociated ganglionic neurons. OP-1 had only a limited stimulatory effect on neuronal survival. However, OP-1 combined with either neurotrophin-3 (NT-3, a relative of nerve growth factor) or glial cell line-derived neurotrophic factor (GDNF) potentiated neuronal survival three- to fourfold. We also show that OP-1 strongly potentiates nerve fiber outgrowth from ganglia stimulated with NT-3 or GDNF. Signaling by BMPR-II in neurons may potentiate the tyrosine kinase pathway activated by NT-3 and GDNF. The data suggest that morphogenetic proteins may modulate neurotrophic activities during neuronal development and plasticity. *J. Neurosci. Res.* 53:559–568, 1998. © 1998 Wiley-Liss, Inc.

Key words: serine/threonine kinase receptors; bone morphogenetic protein receptor type II; BMPR-II; osteogenic protein-1; OP-1; neurotrophin-3; NT-3; glial cell line-derived neurotrophic factor; GDNF

INTRODUCTION

Development of the nervous system requires cell signaling by a series of nerve growth factors and their cell surface receptors. Two major classes of growth factor-responsive transmembrane kinase receptors have been characterized. The first is the tyrosine kinase receptor type (Hanks and Quinn, 1991), which includes the platelet-derived growth factor (PDGF) receptor, the fibroblast growth factor (FGF) receptor, the Trk family (composed of kinase receptors for the neurotrophins [Lewin and Barde, 1996]), and Ret (i.e., the signaling receptor for glial cell line-derived neurotrophic factor

[GDNF; Trupp et al., 1996]). The second major class of growth factor receptors are serine/threonine kinases (Hanks and Quinn, 1991), which mediate signaling by the majority of members within the transforming growth factor (TGF)- β superfamily (Burt and Law, 1994; Griffith et al., 1996); this group includes activins, growth/differentiation factors (GDFs), and bone morphogenetic proteins (BMPs; for review, see Hogan, 1996). These factors have been reported to induce a wide range of effects, including inhibition of cell proliferation; stimulation of extracellular matrix deposition; and promotion of cell differentiation, bone formation, and repair processes. These factors are also important in the patterning of the early embryo. Activin serves a dorsalizing function, and BMPs ventralize the vertebrate embryo (Hogan, 1996; Graff, 1997). Moreover, a potential role for the TGF- β superfamily members in neuronal development, plasticity, and maintenance has also been suggested (Kriegstein et al., 1995; Furuta et al., 1997).

TGF- β and relatives control cell fate by regulating expression of genes encoding key components of cell phenotype. They serve as adapters to specifically engage membrane receptors in heterooligomeric complexes of type I and II serine/threonine kinase receptors. Ligand specificity resides with the type II receptors that are constitutively active kinases. On heterooligomerization, they phosphorylate the type I receptors in a glycine-rich juxtamembrane domain. This activated form of the type I receptor propagates the signal into the cell (ten Dijke et al., 1996) through activating specific cytosolic Smad proteins (Massagué et al., 1997) by phosphorylation (for review, see Massagué, 1996).

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Previously, we have shown that the expression of the serine/threonine kinase receptors types I and II are expressed throughout the nervous system, including many regions of the brain, in type-specific patterns that are strictly regulated during development and on lesion of the adult brain (Bengtsson et al., 1995; Lorentzon et al., 1996; Söderström et al., 1996; Lewén et al., 1997). This expression indicates important roles of the TGF- β superfamily factors—particularly in the BMP subgroup—in the nervous system during development and in the adult organism (Lewén et al., 1997; for review, see Ebendal et al., 1998). These proteins may interact with the neurotrophins to establish the organization of the nervous system. Indeed, Lein et al. (1995) reported that osteogenic protein-1 (OP-1, also known as BMP-7) stimulated dendritic outgrowth in rat sympathetic neurons in culture, with nerve growth factor (NGF) as a necessary cofactor. Lein et al. found that OP-1 distinctly increased the number of dendrites over 10 days in culture. OP-1, BMP-2, and BMP-4 also induce adrenergic differentiation, notably induction of tyrosine hydroxylase and transcription factor Mash1 expression in trunk neural crest stem cells (Reissmann et al., 1996; Shah et al., 1996; Varley and Maxwell, 1996).

We used a low-stringency, degenerate PCR strategy to isolate receptor sequences expressed in embryonic ganglia. Besides a number of well-known receptors (Trk's, FGF receptors, PDGF receptor type A, and type I and type II serine/threonine receptors), we also identified a then-unknown sequence abundantly expressed in ganglionic neurons (deposited in GenBank under account no. Y11870). Subsequent reports by other groups described the same sequence in human and chicken (designated BMPR-II and BRK-3, respectively) as the bone morphogenetic protein receptor type II (BMPR-II; Kawabata et al., 1995; Kawakami et al., 1996). Because human BMPR-II was shown to bind OP-1, we considered it of interest to test this ligand on primary neurons obtained from the chicken embryo. Thus, bioassays of neurotrophic activities of OP-1 and other factors were performed using explanted ganglia in collagen gels (Ebendal, 1989) or dissociated neurons from such ganglia; the stimulation of nerve fiber growth and neuron survival, respectively, were examined (Ebendal, 1987, 1989). Such bioassays allow for distinction among NGF; its relatives neurotrophin-3 (NT-3; Ernfors et al., 1990), brain-derived neurotrophic factor (BDNF), and neurotrophin-4 (NT-4; Hallböök et al., 1995); and other neurotrophic factors such as CNTF and GDNF (Ebendal et al., 1995). With the use of a set of different ganglia (sympathetic, parasympathetic and other autonomic, and sensory), the activity profiles of specific neurotrophic factors can be mapped and ciliary neurotropic factor used to identify various growth factors or to predict their actions on other neurons

(Ebendal et al., 1995)—from the brain, spinal cord, or retina, for example—that carry similar receptors for neurotrophic proteins (Vazquez and Ebendal, 1991; Ebendal et al., 1995; Hallböök et al., 1995). OP-1 was tested in this system at different concentrations, either alone or in combination with established neurotrophic factors. We find that OP-1 alone has limited stimulatory effects on neuronal survival, whereas the neurotrophic effects of NT-3 and GDNF were distinctly potentiated in the presence of OP-1.

MATERIALS AND METHODS

In Situ Hybridization

Four different *in situ* oligonucleotide probes were designed as follows: 1) Chicken BMPR-II, 5'-TCTT-TAAGTGATCTCACAGCCAAGCTGTTCTCTTT CCA TGCTTCTGGG-3', corresponding to amino acid residues FPEAWKENSLAVRSLKETIED in the kinase subdomain X of the chicken BMPR-II cloned here (GenBank accession no. Y11870). The position of this oligoprobe is homologous to nucleotides 244–291 of our sequence and nucleotides 1,586–1,633 in the chicken BRK-3 sequence (AB006785). 2) Chicken *Ret*, 5'-TTGACATAG-GAATCTTCTTCATACACATCACGGGATAGGCCA AAATCA-3', between kinase subdomains VII and VIII, nucleotides 2,677–2,724 of entry Z49898 GenBank. 3) Chicken TrkA, 5'-GGGCAGCATGGTCCGACCGC-CCACCCGGTAGTAGTCGGTGTAGAT-3' (overriding the potential kinase insert site found in TrkC), amino acids IYSTDYY(R/V)GGRTMLP, between kinase subdomains VII and VIII. See Bäckström et al. (1996) for our sequence of chicken TrkA (GenBank accession no. X93581). 4) Chicken TrkC, 5'-AGGCAGCATGGTGT-GTCCTCCAACCCTGTAGTAATCAGTGTAGAC-3', amino acids VYSTDYY(R/V)GGHTMLP, between kinase subdomains VII and VIII, residues 682–697 of the chicken TrkC, nucleotides 2,084–2,131 in the sequence of Okazawa et al. (1993).

The labeling reaction included 5 μ l of 5 \times Cobalt-reaction buffer, 2 μ l of probe (0.005 OD (optical density at 260 nm), where 1 OD = 20 μ g/ml), 8 μ l of 35 S-dATP (1,000 mCi/ml), 2.5 μ l of transferase (TdT; International Biotechnologies, Inc.), and water to a final volume of 25 μ l. It was incubated at 37°C for 1.5 hr. The labeled probe was purified through a NENSORB-20 column (DuPont-NEN Products NLP-022) and equilibrated with 3 ml of 0.1 M Tris-HCl (pH 8.0). The probe was eluted with 0.5 ml of 20% RNase-free EtOH. Two or three drops were collected in 1.5-ml tubes until the column was dry. The hybridization solution used consisted of 0.9 μ l of stem cocktail (5 ml of formamide, 2 ml of 20 \times standard saline citrate [SSC], 0.1 ml of 100 \times Denhardt's solution, and 0.5 ml of 20% sarcosyl [Sigma, St. Louis, MO]), 1.0 ml of 0.2 M phosphate buffer (pH 7.0), 1.0 g of dextran

sulfate, 5.5 μ l of yeast transfer RNA (ytRNA) (50 μ g/ μ l), 50 μ l of salmon sperm DNA (10 μ g/ μ l), 33 μ l of labeled probe, and water to adjust for 10⁶ cpm per section (100 μ l volume). Additionally, just before use, dithiothreitol was added to 60 mM. The tissue slides were incubated overnight at 42°C, washed in 1× SSC at 56°C, dehydrated with graded ethanol (60%, 95%, and 99%), and left to air dry. Then, the slides were dipped in film emulsion (Kodak NTB-2) and incubated for 6 weeks before being developed, counterstained, and mounted.

Bioassays

Two forms of OP-1 were tested: a soluble form (lot 28-003; Creative Biomolecules, Inc., Hopkinton, MA) and a mature form. We also tested the buffer of the soluble form (25 mM arginine, 150 mM NaCl, pH 9, 0.1% Tween 80). GDNF was from PeproTech (Rocky Hill, NJ), and NT-3 and recombinant human activin A were from Austral Biologicals (San Ramon, CA). Mouse β -NGF was prepared according to established routines (Ebendal et al., 1984). In some experiments, we also tested hepatocyte growth factor (HGF; Sigma) and stem cell factor (SCF; Calbiochem, La Jolla, CA).

A series of peripheral ganglia were dissected from E9 chickens (Ebendal, 1989). Sympathetic ganglia were taken from the lumbar region, the ciliary ganglion from the orbit, dorsal root ganglia from the lumbosacral region, and the nodose ganglion from the vagus nerve cranial to the heart. The ganglia were kept intact and placed in a collagen matrix (Ebendal, 1989). For other assays, the ganglia were dissociated into single neurons and spread in a thin collagen gel at a density of 2,000 neurons/cm² (Ebendal, 1987, 1989, Ebendal et al., 1995). In both instances, the gels were supplemented with equal volumes of Eagle's Basal Medium with 1% fetal calf serum. Control cultures consisted of this medium, sometimes supplemented with the OP-1 buffer at appropriate concentrations to match the OP-1 dilution. Cultures also received OP-1, NT-3, GDNF, or NGF alone or in combinations at a series of concentrations. Cultures were incubated at 37°C with 5% CO₂ and examined in an inverted microscope after 2, 4, and 6 days. The 6-day cultures were fed with fresh medium and growth factors at day 4 of incubation. Whole ganglia were examined under dark-field illumination, whereas dissociated neurons were observed using phase-contrast optics. Surviving cells were counted in a strip across the plate to calculate survival relative to the number of seeded neurons.

RESULTS

BMPR-II Expression in Chicken Ganglionic Neurons

Analysis of sequences derived from the PCR of cDNA prepared from E9 chicken sympathetic ganglia

indicated a high BMPR-II expression. Indeed, *in situ* hybridization using an oligonucleotide probe for chicken BMPR-II revealed that BMPR-II is highly expressed in nervous tissues of the embryo. At E4, labeling was present over the neuroepithelium of the neural tube and the forming dorsal root ganglia. At E9, labeling was also strong over the paravertebral sympathetic ganglia (Fig. 1A,B).

In contrast, we have previously shown no obvious labeling of this ganglion for TrkB, the NT-3 receptor (Fig. 1C) (Bäckström et al., 1997). However, NT-3 has been reported to exert effects upon binding to TrkA (Davies et al., 1995; Fagan et al., 1996; Belliveau et al., 1997), which is highly expressed in the E9 sympathetic ganglia (Fig. 1D; Bäckström et al., 1996). Furthermore, mRNA encoding Ret, the GDNF receptor (Trupp et al., 1996), is expressed at low-to-modest levels in the E9 sympathetic ganglion (data not shown).

We also examined the expression of kinase receptors in neuronal populations of the dorsal root ganglion. One interesting finding is that the BMPR-II was evenly distributed throughout neurons in the ganglion (Fig. 1E). The mRNA for activin receptor type II (ActR-II) also shows labeling throughout the entire ganglion. TrkB is distinctly expressed by large ventrolateral neurons (Williams and Ebendal, 1995; Bäckström et al., 1997); neurons expressing TrkA mainly occupy a dorsomedial position (Bäckström et al., 1996). Ret mRNA is localized to neurons mainly positioned as a band across the lower one-third of the ganglion. The early expression of Ret mRNA is localized to chicken sympathetic, dorsal root, and trigeminal ganglia and to spinal cord motor neurons (Nakamura et al., 1996). Furthermore, we found distinct BMPR-II expression in neurons of the E9 trigeminal ganglion (Fig. 1F). Modest BMPR-II expression was also found in the neurons of the nodose ganglion. Stronger labeling of the BMPR-II transcript were found in the E9 ciliary ganglion (not shown). The expression was already distinct at E6. Many neurons in the ciliary ganglion, examined at E9, also expressed Ret.

Neurotrophic Activities Potentiated by OP-1 in Sensory and Autonomic Ganglia

Given the fact that the mRNA for BMPR-II is distinctly expressed in sympathetic and sensory ganglia, we wanted to test whether one of its reported ligands, OP-1 (Liu et al., 1995; Rosenzweig et al., 1995), had biological effects on neurons from these tissues. To test for survival effects of the factor, we cultured dissociated sympathetic neurons in the presence or absence of the trophic factors alone and in combination with OP-1 (Fig. 2). Figure 3 shows the results of neuron survival after 2 and 6 days of culture. The survival induced by OP-1 (50 ng/ml) after 6 days of culture was only slightly

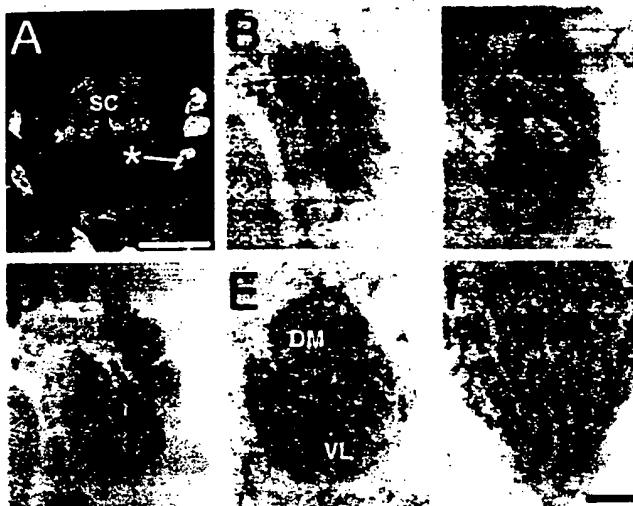


Fig 1. **A:** Cross section of an E9 chicken showing labeling of mRNA for BMPR-II in the spinal cord (SC), dorsal root ganglia, and sympathetic ganglia (examined by using dark-field illumination). Some mesenchymal tissue is also labeled. Scale bar = 0.5 mm. **B:** Dense BMPR-II labeling in E9 sympathetic ganglion. **C:** No expression of TrkC mRNA was found in the sympathetic ganglion. **D:** As in B, but with a probe giving positive labeling for TrkA. **E:** BMPR-II expression in the dorsal root ganglion of E9 chicken. Labeling is present in neurons throughout the ganglion, but the strongest signal is found large ventrolateral (VL) neurons. Neurons expressing TrkA mainly occupy a dorsomedial (DM) position. **F:** Distinct expression of BMPR-II mRNA in neurons of the E9 chicken trigeminal ganglion. Asterisk (*) indicates the sympathetic ganglion (A-D). Scale bar = 0.5 mm (A); scale bar = 0.1 mm (B-F).

(but nevertheless significantly) higher than survival in control medium (see Fig. 3). About 40% of cultured neurons exposed to NT-3 at 10 ng/ml (Fig. 2C) were rescued after 2 days (Fig. 3A), whereas 100% of those exposed to NGF survived after 2 days. The NT-3 effect did not persist at day 6 of culture (Fig. 3B). Interestingly, the combination of OP-1 and NT-3 improved neuron survival significantly (Fig. 2C), rescuing 75% of the neurons after 2 days of culture and 50% after 6 days (Fig. 3). The survival effect of the combination of OP-1 and NT-3 was as good as that of NGF after 6 days of culture (Figs. 2D, 3B). Also, the combination OP-1 and GDNF (both 50 ng/ml) gave significantly higher survival rates among sympathetic neurons after 2 and 6 days than either factor alone (Fig. 3). Similar findings were obtained when the neurons were allowed to grow on laminin substrates (not shown) rather than in collagen gels.

To test OP-1 stimulation of neurite outgrowth, we also used a whole-ganglia explants bioassay (Ebendal, 1989). Stimulation of E9 chicken sympathetic ganglia was poor for OP-1, tested at the range of 0.1 to 1,000 ng/ml (data not shown). Moreover, OP-1 failed to stimu-

late the fiber formation in sympathetic ganglia when tested at younger ages (E4.5). NT-3 stimulated only limited fiber outgrowth from the explanted sympathetic ganglia (Fig. 4A; Ernfors et al., 1990). However, the combination of OP-1 and NT-3 caused the E9 ganglion to form a dense halo of outgrowing neurites in culture (Fig. 4B), suggesting a synergistic effect. The effects were obvious after 2 days in culture and persisted beyond 4 days of incubation. Adding only 2 ng/ml of NT-3 with 50 ng/ml of OP-1 resulted in slightly less dense fiber outgrowth.

We tested other peripheral ganglia in similar assays. Effects of OP-1 similar to those observed in sympathetic neurons were noted in explanted dorsal root ganglia (data not shown), where NT-3 alone (10 ng/ml) seemingly had a better effect on outgrowing neurites than OP-1 (50 ng/ml). The potentiating effect of these two factors in combination is striking after 2 days in culture. Similar but less prominent effects were obtained with OP-1 and GDNF together (both 50 ng/ml). Ciliary ganglia did not extend neurites in the presence of only OP-1 (Fig. 4C). However, as reported previously, NT-3 (Ernfors et al., 1990) or GDNF (Fig. 4D) (Ebendal et al., 1995) initiated only limited nerve fiber outgrowth. In contrast, NT-3 and OP-1 in combination stimulated the formation of robust fiber halos consisting of thick neurite fascicles from the E9 ciliary ganglia (data not shown). Lowering the NT-3 concentration to 2 ng/ml and adding 50 ng/ml OP-1 reduced the fiber outgrowth but still resulted in a distinct fiber halo surrounding the ganglion. Notably, GDNF and OP-1 together gave a dense fiber halo of ciliary nerve fascicles after 2 days of culture (Fig. 4E). In contrast, NGF and OP-1 together did not stimulate any fiber outgrowth in the ciliary ganglia.

We also studied the effects of the combined trophic factors on the nodose ganglion. OP-1 alone did not evoke any fiber outgrowth. Although NT-3 normally stimulates fiber formation in this ganglion (Ernfors et al., 1990), OP-1 further enhanced neurite outgrowth after 2 and 4 days of incubation. This effect was confirmed by repeated blind tests involving independent observers (data not shown). Finally, we tested survival effects on dissociated ciliar neurons. OP-1 significantly potentiated survival among neurons responding to GDNF (Fig. 5); this finding is in agreement with the co-expression of BMPR-II and Ret in the neurons of this ganglion.

The stimulatory action by OP-1 on NT-3-induced neuron survival was not mimicked by activin A (tested from 1 to 1,000 ng/ml), nor did we find that activin A or TGF- β 1 alone stimulated survival or fiber outgrowth in any of the ganglia tested (Ebendal et al., 1995). If anything, activin A seemed to inhibit rather than potentiate the weak NT-3-stimulated fiber outgrowth in sympa-

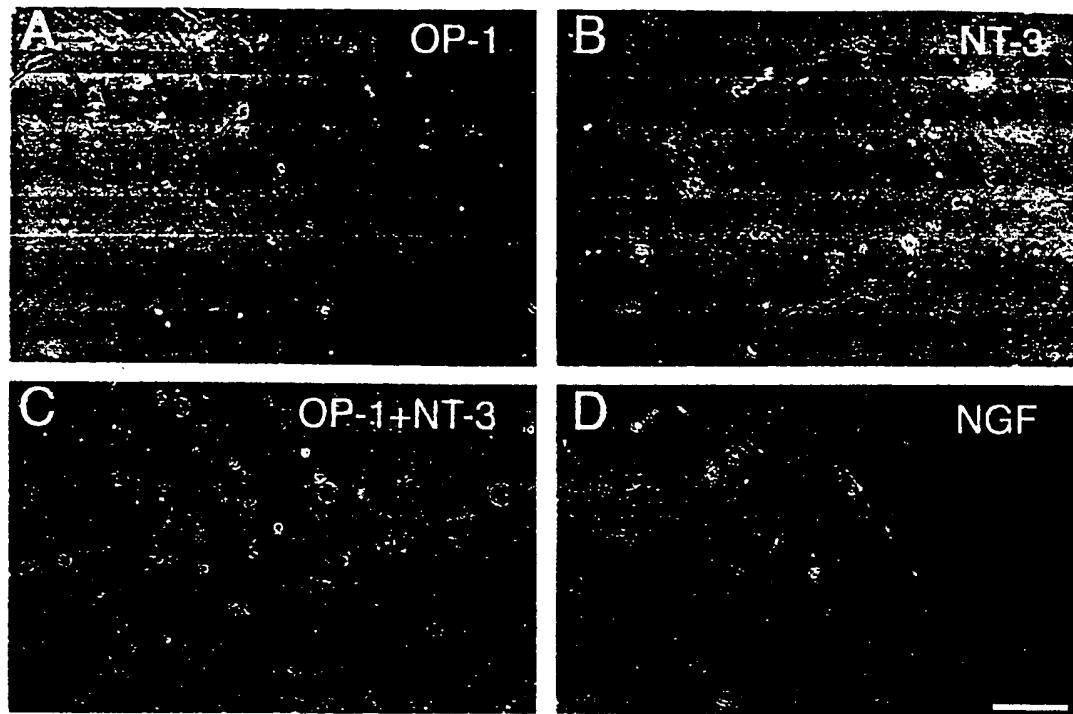


Fig 2. Phase-contrast micrographs show the survival of sympathetic neurons after 6 days in a collagen gel culture. A: With OP-1 (50 ng/ml), a few neurons extending neurites can be seen. B: More neurons survive in the presence of NT-3 at 10 ng/ml.

C: Numerous neurons surviving in the presence of OP-1 and NT-3 (50 and 10 ng/ml, respectively). The neurons develop long neurites. D: Sympathetic neuron survival and neurite formation in the presence of NGF (5 ng/ml). Scale bar = 0.5 mm.

thetic ganglia (data not shown). Moreover, the OP-1 buffer (pH 9) failed to exert any potentiating effect on the NT-3 responses in sympathetic, ciliary, or nodose ganglia. OP-1 did not result in fiber outgrowth (data not shown) when tested on E9 ganglia with HGF or SCF, acting on the tyrosine kinase receptors Met and Kit, respectively (Hirata et al., 1995; Wong et al., 1997).

Trk and Ret Expression Not Upregulated in Response to OP-1

To rule out the possibility that OP-1 merely upregulates the expression of Trk and Ret receptors, we used quantitative *in situ* hybridization to study cultured ganglia. Ganglia were grown in presence of either NT-3 alone or NT-3 and OP-1 together, and then TrkA and TrkC expression was examined in X-ray films by densitometry (Söderström et al., 1996). For examination of Ret, ciliary ganglia were grown with GDNF alone or with GDNF and OP-1 together. We found no significant upregulation of TrkA, TrkC, or Ret under these conditions as determined by the Mann-Whitney U-test (data not shown).

DISCUSSION

The present results demonstrate that specific combinations of growth factors can distinctly potentiate neuro-

trophic effects in subsets of neurons. We found strong stimulation by OP-1 on neurons responding to NT-3 and GDNF, in terms of both survival and nerve fiber outgrowth. OP-1 signals via serine/threonine kinase receptors (Liu et al., 1995; Rosenzweig et al., 1995). We show that one such receptor, BMPR-II, was expressed by the neurons used in our assays. In contrast, both NT-3 and GDNF are known to activate tyrosine kinase receptors (Lewin and Barde, 1996; Trupp et al., 1996). The observations imply that dual signaling via tyrosine and serine/threonine kinase receptors potentiates neurotrophic effects. Such an interplay may well occur during normal development of the nervous system and later contribute to neuroplasticity.

The effects exerted by OP-1 were not mimicked by activin A, even though the ganglionic neurons expressed the ActR-II receptor. We believe that ActR-II is less likely to be involved in the potentiating effects, despite the fact that OP-1 at high concentrations has been shown to activate this receptor (Yamashita et al., 1995).

NT-3 Probably Acts on Sympathetic Neuron TrkA Receptors

It is perhaps unexpected that NT-3, but not NGF, has the ability to synergize with OP-1 to enhance the

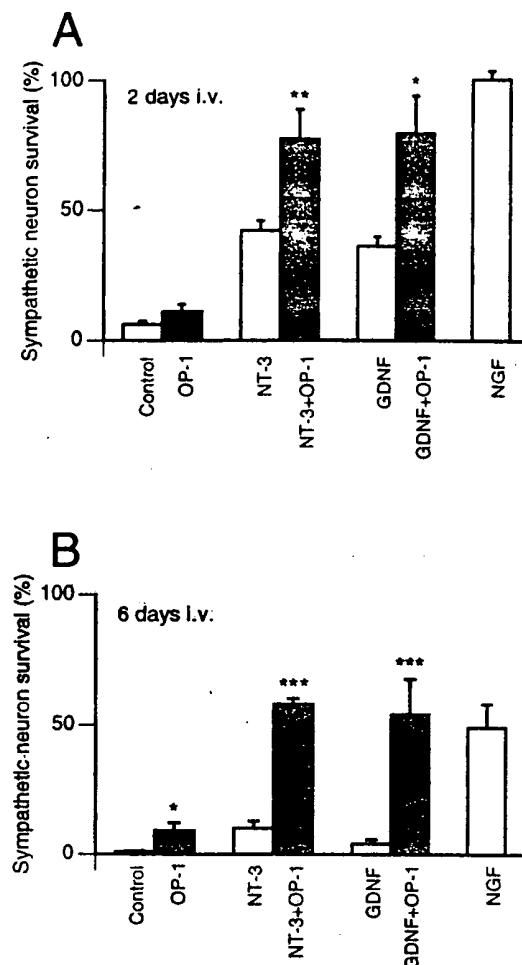


Fig 3. Survival of dissociated sympathetic neurons grown in collagen gels. **A:** Survival after 2 days in vitro (i.v.). NGF (5 ng/ml) rescues 100% of the neurons seeded at 2,000 cells/cm² (Ebendal et al., 1995). Low survival rates were seen in the presence of control medium or OP-1 only (50 ng/ml); differences between these groups were not statistically significant. NT-3 (2 ng/ml) enhanced survival above background, as did GDNF (50 ng/ml; Ebendal et al., 1995). In both instances, the effects were markedly enhanced when OP-1 was also present (**P < 0.01 and *P < 0.05, respectively; Mann-Whitney U-test). **B:** Survival after 6 days of culture. OP-1 has a slight survival effect (*P < 0.05) above plain medium (with 1% fetal calf serum present in both instances). OP-1 with NT-3 and GDNF potentiated survival even more marked than after 2 days (**P < 0.001 for both groups). Data are based on 6–10 independent cultures per bar. Bars indicate SEM.

survival and neurite outgrowth of sympathetic neurons. NGF rescues 100% of the sympathetic neurons grown in culture (Ebendal et al., 1995), an effect which is likely to be mediated by the TrkA receptor (Ip et al., 1993; Wyatt et al., 1997) expressed by these neurons (Bäckström et al., 1996). In our studies, OP-1 did not act synergistically

with NGF, even when NGF was added at low concentrations, resulting in low levels of sympathetic neuron survival and neurite outgrowth (comparable to levels observed when 10 ng/ml NT-3 was added to the cultures; Ernfors et al., 1990). Thus, NT-3 does not act as a suboptimal NGF when potentiating the effects of OP-1.

The expression of TrkC mRNA is very low in E9 chicken sympathetic neurons (Hallböök et al., 1995; Bäckström et al., 1997), but TrkA mRNA is highly expressed (Hallböök et al., 1995; Bäckström et al., 1996). Neuronal survival evoked by NT-3 might be mediated by the TrkA receptor, as suggested by Ip et al. (1993). NGF dependence during the development of the postmitotic sympathetic neurons that express TrkA has been suggested on the basis of culture experiments (Birren et al., 1993; DiCicco-Bloom et al., 1993). Such a model is partially supported by analyses of the number of sympathetic neurons surviving in the superior cervical ganglion (SCG) of mice bearing null mutations in the TrkA or TrkC genes (Fagan et al., 1996). This study showed that elimination of TrkC did not reduce the number of sympathetic neurons, in contrast to studies of NT-3 deficient mice that showed a 50% reduction in the number of sympathetic neurons in the SCG (Ernfors et al., 1994; Fariñas et al., 1994). Moreover, Fagan et al. (1996) demonstrated an absolute requirement for the TrkA receptor in perinatal development of sympathetic neurons.

Again, the combined results of these studies indicate that TrkA in vivo may serve as a receptor for both NGF and NT-3 (Fagan et al., 1996), as shown in cellular contexts *in vitro* (Ip et al., 1993). Direct evidence for NT-3 signaling via TrkA was obtained by Davies et al. (1995), who studied survival responses by NT-3 (at concentrations of 100–1,000 ng/ml) in cultures of sympathetic neurons obtained from mouse embryos homozygous for a TrkC null mutation. Wyatt et al. (1997) also showed that mouse SCG neurons depend on NT-3 only at a late stage of the embryonic period and that catalytic TrkC is not expressed.

A recent study (Belliveau et al., 1997) lends additional support to the idea that NT-3 acts on TrkA to enhance survival and neurite formation in sympathetic neurons. The data indicated that NGF and NT-3 may evoke differential responses by the shared TrkA receptor. Consequently, the potentiating effects evoked by OP-1 and NT-3 in sympathetic neurons are likely to involve the combined activation of BMPR-II and TrkA receptors.

Synergistic Action With NT-3 and GDNF in Restricted Neuron Populations

Our data show that OP-1 has a powerful ability to enhance some of the neurotrophic effects of NT-3 and GDNF. OP-1 alone has limited capacity to act as a

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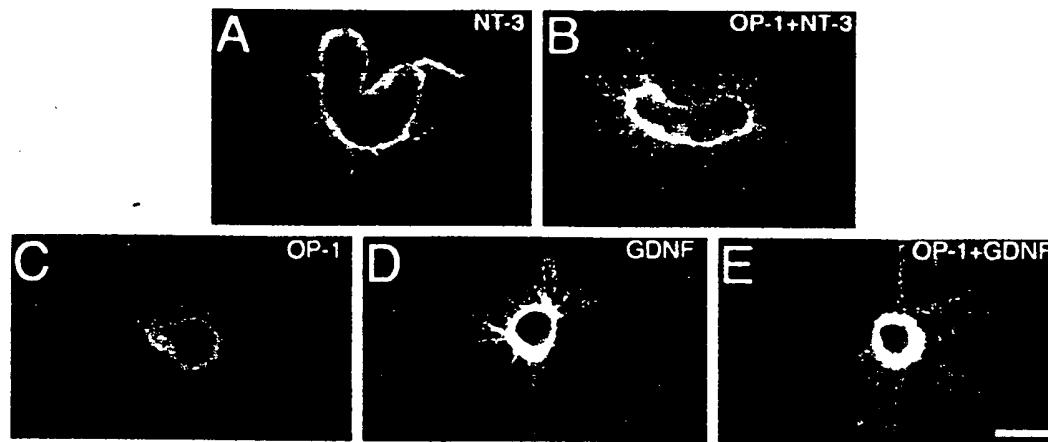


Fig 4. Nerve fiber outgrowth from explanted sympathetic and ciliary E9 ganglia were examined by using dark-field illumination. A: Fiber outgrowth of a sympathetic ganglion in the presence of NT-3 (10 ng/ml). A few fiber fascicles grew from the ganglion in response to NT-3. B: Sympathetic ganglion explant grown in the presence of OP-1 (50 ng/ml) and NT-3 (10 ng/ml). A dense fiber halo penetrating the collagen matrix

surrounding the ganglion has formed. C: The ciliary ganglion shows no response to OP-1 (50 ng/ml). D: The ciliary ganglion cultured with GDNF (50 ng/ml) responded with distinct fiber outgrowth. E: GDNF (50 ng/ml) and OP-1 (50 ng/ml) resulted in a dense halo of outgrowing neurites. All ganglia were incubated for 2 days. Scale bar = 0.3 mm.

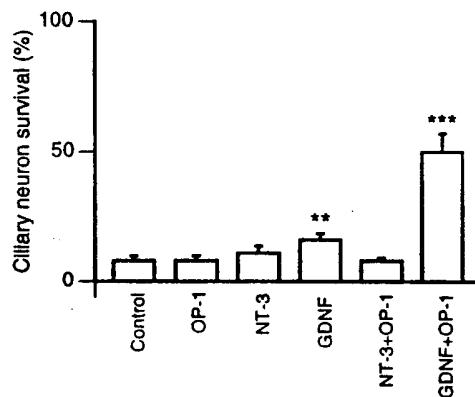


Fig 5. Survival effects of OP-1 on dissociated ciliary neurons. E9 chicken ciliary ganglia were dissociated and the neurons explanted in collagen gels. Surviving neurons were counted under the phase-contrast microscope after 2 days of culture. As shown previously, an extract of the choroid coat of the E18 chicken embryo rescued 100% of the seeded neurons (Ebendal, 1987). OP-1 did not stimulate survival, and NT-3 had only a marginal effect (not statistically significant). GDNF at 50 ng/ml rescued neurons significantly (** $P < 0.01$) above control levels (Ebendal et al., 1995). However, OP-1 and GDNF together potentiated survival and rescued more than one-half of the seeded ciliary neurons (** $P < 0.001$; Mann-Whitney U-test). Data are based on 6–9 cultures per bar. Bars indicate SEM.

neurotrophic factor for the tested peripheral neurons. This limitation may not be valid for all populations of neurons, because OP-1 exerts a strong neuritogenic effect on explants of the embryonic retina without additions of other exogenous trophic factors (Carri et al., 1998). The

synergistic activity of OP-1 is limited to cooperation with the two neurotrophic factors NT-3 and GDNF and is not seen with a number of other related molecules tested in the peripheral neuronal cultures. The effects of combining OP-1 with either NT-3 or GDNF are 3.2- to 4.4-fold higher than the combined effects of each factor tested alone, measured as sympathetic neuron survival after 6 days in culture (Fig. 3B). In the 2-day ciliary neuron cultures, the potentiating effect of OP-1 on GDNF-evoked survival was increased 2.6-fold (Fig. 5).

Synergistic effects by neurotrophic factors have indeed been demonstrated previously. In general, the effects have been more limited than those seen in the present study. CNTF has been shown to synergize with HGF by activating tyrosine kinase receptor Met to promote survival and cholinergic differentiation in cultured motor neurons (Wong et al., 1997). Combining these two factors resulted in an increase 30% higher than expected from merely adding the effects of each factor alone. Furthermore, SCF, acting on tyrosine kinase receptor Kit, acts synergistically with NGF at submaximal concentrations to support the survival of a subpopulation of Kit-positive sensory neurons from the mouse dorsal root ganglion (Hirata et al., 1995). The potentiation was, at best, in the range of 50% higher than the sum of the effects of each factor alone. In contrast, we found that OP-1 in combination with either HGF or SCF did not stimulate ganglia under our experimental conditions (data not shown).

Kriegstein and Unsicker (1996) reported that TGF- β 3, activating a serine/threonine kinase, has no survival

effects on dissociated E8 chicken dorsal root ganglion neurons. However, TGF- β 3 combined with either NT-3 or NT-4 increased neuronal survival 15–27% over the additive effects. In contrast, TGF- β 3 did not act with NGF to raise survival above the level evoked by NGF alone.

In addition, GDF-5 (another member of the TGF- β superfamily), administered with NT-3, enhances the survival of chicken dorsal root ganglion neurons 30% (Farkas et al., 1997).

Separate Signaling Pathways Converge to Potentiate Neurotrophic Effects

In mammalian cells, BMPR-II binds OP-1 and forms heterooligomeric complexes (ten Dijke et al., 1994, 1996) with several type I receptors (activin receptor type I [ActR-I], BMP receptor type IB [BMPR-IB], or, with less efficiency, BMP receptor type IA [BMPR-IA]). Previously, we demonstrated that sympathetic and dorsal root ganglia of the rat embryo express ActR-I (Söderström et al., 1996) but only extremely low levels, if any, of BMPR-IA and BMPR-IB. By homology, we regard it likely that ActR-I is the transducing partner for OP-1/BMPR-II in the chicken ganglia studied in the present study. Thus, one obvious signaling pathway for the OP-1 stimulation is serine/threonine kinase receptor phosphorylation of downstream substrates. The other pathway implied in our findings is by NT-3 or GDNF activation of tyrosine kinase receptors.

Where—and if—these two pathways meet to exert their potentiating neurotrophic action is not known. Shared downstream substrata for phosphorylation and the level of cross-talk between the two pathways remain to be studied. Kretzschmar et al. (1997) showed that BMP and epidermal growth factor signaling converge on Smad-1. In this case, the serine/threonine and tyrosine kinase receptor pathways resulted in differential phosphorylation of separate residues in the Smad protein, leading to opposing functions. It is conceivable that analogous mechanisms account for the potentiating (rather than opposing) effects by dual signaling observed in the present study.

The present findings may suggest the use of OP-1 in situations where nerve cells are severed or degenerating as a result of disease, especially if additional neurotrophic factors can be expected to be produced locally or administered with OP-1. We have demonstrated specific potentiating mechanisms in distinct subsets of peripheral neurons. However, it is likely that similar mechanisms can be found in populations of neurons in the central nervous system. Notably, dopaminergic neurons in substantia nigra express both Ret and BMPR-II receptors (Söderström et al., 1996; Trupp et al., 1996). Another possibility is to use the new knowledge to target the

possibly converging pathways in a dual-signaling system that involves serine/threonine and tyrosine kinase receptors activated by OP-1, NT-3, and GDNF. Thus, downstream entities in the neuronal tyrosine and serine/threonine kinase receptor cascades will be stimulated directly to produce effects similar to those elicited by the extracellular administration of factors that activate these pathways.

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Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: Developmental and topographical expression in the brain

(nerve growth factor family/cDNA/neurotrophic factor/hippocampal neurons/nerve growth factor receptor binding)

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ABSTRACT We have used a pool of degenerate oligonucleotides representing all possible codons in regions of homology between brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) to prime rat hippocampal cDNAs in the polymerase chain reaction. The amplified DNA included a product with significant similarity to NGF and BDNF, which was used to isolate a 1020-nucleotide-long cDNA from a rat hippocampal library. From the nucleotide sequence, a 282-amino-acid-long protein with ≈45% amino acid similarity to both pig BDNF and rat NGF was deduced. In the adult brain, the mRNA for this protein was predominantly expressed in hippocampus, where it was confined to a subset of pyramidal and granular neurons. The developmental expression in brain showed a clear peak shortly after birth, 1 and 2 weeks earlier than maximal expression of BDNF and NGF, respectively. It was also expressed in several peripheral tissues with the highest level in kidney. The protein, transiently expressed in COS cells, was tested on chicken embryonic neurons and readily stimulated fiber outgrowth from explanted Remak's ganglion and, to a lesser extent, the nodose ganglion. A weak, but consistent, fiber outgrowth response was also seen in the ciliary ganglion and in paravertebral sympathetic ganglia. Moreover, the protein displaced binding of NGF to its receptor, suggesting that it can interact with the NGF receptor. Thus, this factor, although structurally and functionally related to NGF and BDNF, has unique biological activities and represents a member of a family of neurotrophic factors that may cooperate to support the development and maintenance of the vertebrate nervous system.

During development of the vertebrate nervous system, a vast overproduction of neurons is compensated for by naturally occurring neuronal death, which is regulated by their targets (1). Within the targets, specific proteins, referred to as neurotrophic factors, are produced in limiting amounts and the release of these proteins is believed to regulate both the timing and the extent of innervation (2).

In the peripheral nervous system, the most well-characterized neurotrophic factor, nerve growth factor (NGF), supports the development of sympathetic and neural crest-derived sensory neurons, and in the adult the maintenance of the sympathetic nervous system is critically dependent on NGF (3, 4). In agreement with a trophic role of NGF for adult sympathetic neurons, the levels of both NGF mRNA and protein correlate with the density of sympathetic innervation (5, 6). NGF mRNA and protein have also been found in the brain, with the highest levels in hippocampus and cerebral cortex, to which the major cholinergic pathways in the brain project (7–10). Basal forebrain cholinergic neurons can be

prevented from dying after axonal transection by addition of NGF (11–15) and they respond to NGF *in vivo* by a marked increase in fiber outgrowth (16).

In addition to NGF, one other protein, termed brain-derived neurotrophic factor (BDNF), has been shown to be present in low amounts (17), secreted from cells (18), and to support survival of embryonic sensory neurons *in vivo* (19). In common with NGF, BDNF supports the survival of neural crest-derived embryonic sensory neurons *in vitro*, but nonoverlapping trophic activities are suggested by the finding that BDNF also supports placode-derived neurons from the nodose ganglia and retinal ganglion cells (20, 21), which are less sensitive to NGF (22, 23). Regulation of neuronal survival *in vivo* in the brain by BDNF has not yet been demonstrated, although its sites of synthesis have recently been mapped by *in situ* hybridization where a high level of labeling was found in hippocampal neurons (24).

NGF is synthesized as a preprotein and the structure of both the precursor and the mature protein has been deduced from cDNA and genomic clones (25, 26). More recently, a genomic clone has been isolated for porcine BDNF (18). Of considerable interest is the finding that the mature BDNF and NGF proteins show striking amino acid similarities, suggesting that they are structurally related and may be members of a family of neurotrophic factors (18).

In this study, we report on the cloning and expression of an additional member of the NGF family. Due to its restricted expression in the brain, being mostly confined to a subset of pyramidal and granular neurons in the hippocampus, we have named this protein hippocampus-derived neurotrophic factor (HDNF).

MATERIALS AND METHODS

RNA Preparation, Molecular Cloning, and DNA Sequencing. Polyadenylated RNA [poly(A)⁺] was prepared as described (27). For cloning, rat hippocampus poly(A)⁺ RNA (5 µg) was used as a template for synthesis of single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase (Pharmacia). Six separate mixtures of 28-mer oligonucleotides representing all possible codons corresponding to the amino acid sequence KQYFYET (5'-oligonucleotide) and WRFIRID (3'-oligonucleotide) were synthesized on an Applied Biosystems A381 DNA synthesizer. The 5'-oligonucleotide contained a synthetic EcoRI site and the 3'-oligonucleotide contained a synthetic HindIII site. Each mixture of oligonucleotides was then used to prime the amplification of hippocampal cDNA (25 ng) by the polymer-

Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; HDNF, hippocampus-derived neurotrophic factor; PCR, polymerase chain reaction.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34643).

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ase chain reaction (PCR) (Gene Amp, Perkin-Elmer/Cetus). PCR products of the expected size [182 base pairs (bp) including primer and restriction site] were isolated on an agarose gel and cloned in plasmid Bluescript KS⁺ (Stratagene) followed by nucleotide sequence analysis by the dideoxynucleotide chain-termination method (28). The insert from one clone showing ≈60% nucleotide sequence similarity to both rat NGF and pig BDNF was then used to screen a cDNA library in λgt10 from rat hippocampus constructed with a cDNA synthesis kit (Pharmacia). Screening of 1.2 × 10⁶ independent cDNA clones from the primary library (29) yielded seven positive clones. One clone with a 1020-bp-long insert was sequenced in its entirety on both strands by the chain-termination method.

RNA Blot Analysis. Poly(A)⁺ RNA (20 μg) was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde and was transferred to a nitrocellulose filter. The filter was hybridized to a 355-bp fragment from the 3' end of HDNF mRNA (nucleotides 665–1020 in Fig. 1), isolated by PCR using one specific primer together with an oligo(dT) primer as described by Frohman *et al.* (30). The fragment was labeled with [α -³²P]dCTP by nick-translation to a specific activity of $\approx 5 \times 10^8$ cpm/μg. Hybridization was carried out as described (27) followed by washing at high stringency and exposure to Kodak XAR-5 films. The same filters were boiled for 5 min in 1% glycerol, followed by hybridization first to a 185-bp PCR fragment from rat BDNF corresponding to amino acids 183–239 of pig BDNF (18), then to a 770-bp *Bst*E2/*Pst*I fragment from the 3' exon of rat NGF (31), and finally to a 1.5-kilobase (kb) *Pst*I fragment from a mouse α -actin cDNA (32). Appropriate exposures of all autoradiograms were quantified with a Shimadzu CS-9000 densitometer.

In Situ Hybridization. Cryostat sections (14 μm) from fresh-frozen adult Sprague-Dawley rat brain were processed and used for *in situ* hybridization with deoxyadenosine [α -[³⁵S]thio]triphosphate 3'-end-labeled probes as described (33). To detect HDNF-specific mRNA, a 50-mer oligonucleotide complementary to nucleotides 667–717 in Fig. 1 was used. For BDNF-specific mRNA, a 50-mer oligonucleotide complementary to rat BDNF mRNA, corresponding to nucleotides 748–798 in pig BDNF (18), was used.

Expression of HDNF Protein, Assays of Biological Activities, and Binding to the NGF Receptor. The 1020-bp HDNF cDNA insert in λgt10 was amplified by PCR using λgt10 sequencing primer and reverse primer (Clontech). The amplified DNA was treated with T4 polynucleotide kinase and 10-mer *Xba*I linkers were ligated, followed by cloning of the fragment into the *Xba*I site of pXM (34). COS cells grown to $\approx 70\%$ confluence were transfected with 20 μg of the indicated plasmid construct per 10-cm dish by the DEAE dextran-chloroquine method (35). A plasmid expressing the β -galactosidase gene (pCH110, Pharmacia) was transfected in parallel and β -galactosidase activity was measured in cytoplasmic extracts as a control of transfection efficiency. The conditioned medium from transfected cells (36) was then collected and assayed for stimulation of neurite outgrowth from embryonic chicken ganglia as described (37). Conditioned medium from transfected cells was tested for binding to the NGF receptor on PC12 cells as described (36).

RESULTS

Molecular Cloning and Structure of HDNF. A 1020-bp cDNA clone was isolated. Nucleotide sequence analysis of this clone showed an open reading frame encoding a 282-amino-acid-long protein (Fig. 1). The C-terminal part of this protein contained a potential cleavage site for a 119-amino acid protein with 57% amino acid similarity to both rat mature NGF and pig mature BDNF. Included in this similarity were all six cysteine residues, involved in formation of disulfide bridges, and an overall identity to NGF and BDNF of 68 and

1	V D V P G N S H T D A M V T S A T I L Q	20
1	GTGACGCTCCGGAAATAGTCATACGGATGCCATCGTTACTCTGCCACGATCTACAG	60
21	V N K V M S I L F Y V I F L A Y L R G I	40
21	GTGACAAGGTGATGTCATCTGTTATGTGATATTCTGCTTATCTCCATCTACAT	120
41	Q G N N M D Q R S L P E D S L N S L I I	60
121	CAAGCCAACACATGGATCAAAGGACTTGGCCAGAAGACTCTCTCAATTCCCTCATATTAC	180
61	K L I Q A D I L K N K L S K Q M V D V K	80
181	AAGTTGATCCAGCGGGATATCTGAAAACAAGCTCTCAACGGAGATGGATGTTAAG	240
81	E N Y Q S T L P K A E A P E Q G E	100
241	GGAAAATACAGAGCACCCCTGCCCAAGCAGGGACCAGAGAACAGAGCAGGGAGAG	300
101	A T R S E F Q P M I A T D T E L L R Q Q	120
301	GCCACCAAGTCAGAATTCCAGCGATGATTGCAACAGACAGAACAGAACTACAGCGAACAG	360
121	R R Y N S P R V L L S D S T P L E P P P	14
361	AGACGCTAACATTACCCCCGGGCTCTGCTGAGTGACAGCAGCCCTTGGAGCCCCCTCC	420
141	L Y L M E D Y V G N P V V T N R T S P R	16
421	TTTATCTAATGGAGATTATGTGGCAACCCGGTAAACCAATAAGAACATCACACCGG	480
161	R K R Y A E H K S H R G E Y S V C D S E	18
481	AGGAACAGCTATGCAGAGCATAAGAGTCACCGAGGAGAGTACTCGATGTGACAGTGTGAG	540
181	S L W V T D K S S A I D I R G H Q V T V	20
541	AGCCTGTGGGTGACCGACAAGCTCAGGCAATTGACATTGGGGACACCAGGTTACAGTG	600
201	L G E I K T G N S P V K Q Y F E Y E T R C	22
601	TGAGGAGATCAAAACCGGCAACTCTCTGTGAAACATATTGTAAGACAGGAGTGT	660
221	K E A R P V K N G C R G I D D K H W N S	24
661	AAAGAACGCCAGGGCAGTCAAAAACGGTTGCAGGGGATTGATGACAAACACTGGAACTCT	720
241	Q C K T S O T Y V R A L T S E N N K L V	26
721	CAGTGCACAAACGGCAAAACCTAGCTCCGAGCACTGACTCAGAAAACAACAACTCGTA	780
261	G W R W I R I D T S C V C A L S R K I G	28
781	GGCTGGCCGTGGATCGAATAGACACTCTCTGTGTGTCCTGTCAGAAAATTCGGA	840
281	R T End	28
841	AGAACATGAATTGGCATCTGCCCCACATATAATTATTACTTTAAATTATGATGATG	900
901	ATGAGCATATAATGTTATATGTTATATATAAGTTGACCTTATTTATTA	960
961	ACTTCAGCAACCCCTTACAGTATATAACCTTTTCATATCGGGCTGCTCAAAAAAA	1020

Fig. 1. Nucleotide sequence and deduced amino acid sequence of rat HDNF. Arrow indicates the presumptive start of mature HDNF. A consensus sequence for N-glycosylation is shown in the box, at the stretches of adenosine at the end of the sequence show the poly(A) tail. Vertical bar shows an exon/intron boundary present in the rat NGF gene (31). Stars indicate potential translation start site.

67 residues, respectively, was seen. The prepro part of the protein showed weak, but significant, homology to NGF. A potential N-glycosylation site located nine amino acids from the start of the mature protein was also conserved between the three proteins.

Expression of HDNF mRNA in Peripheral Tissues. A 1.3-kb HDNF-specific mRNA was detected in several adult rat peripheral tissues, with the highest level in kidney (data not shown). Densitometer scanning of autoradiograms from three independent experiments showed that the spleen and heart contained approximately 6- and 8-fold lower levels respectively, compared with kidney. Lower levels were also found in the adrenal gland, ovary, muscle, and liver. Hybridization of the same filter to a rat NGF probe revealed that the amounts of HDNF mRNA in heart and spleen were comparable to the level of NGF mRNA in these tissues.

Developmental Expression of HDNF and BDNF in Rat Brain. In the developing brain, HDNF mRNA was detected already at embryonic day 15, the earliest time point tested (Fig. 2a). A sharp increase was seen at birth and maximum levels were found at postnatal day 4. At 3 weeks of age, the amount had decreased to adult levels. Densitometer scanning of autoradiograms from two independent experiments showed that adult levels were 15-fold lower than at postnatal day 4. The level in the adult brain was lower than in kidney but was comparable to the level in heart. A 1.4-kb BDNF mRNA was first seen at embryonic day 19, with a peak level at 2 weeks of age, which was 10-fold higher than the amount in adult brain (Fig. 2a). A 4.0-kb BDNF mRNA was also seen and the developmental and regional expression of this mRNA was the same as for the 1.4-kb mRNA.

Regional Distribution of HDNF and BDNF mRNA in Adult Rat Brain. The distribution of HDNF mRNA in the ad-

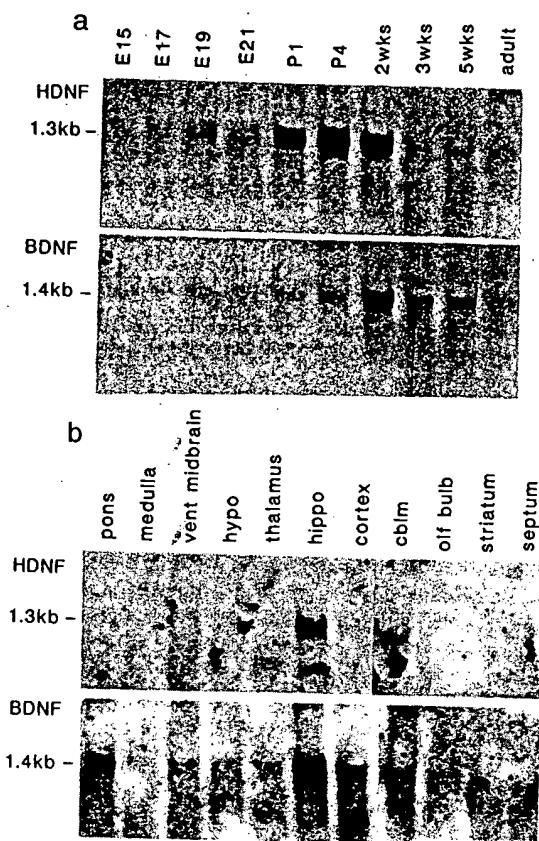


FIG. 2. Developmental and regional expression of HDNF and BDNF mRNA in rat brain. (a) Poly(A)⁺ RNA (20 µg per slot) isolated from Sprague-Dawley rat brain at the indicated developmental stages was hybridized to the indicated probes (HDNF and BDNF). Adult rats were 12 weeks old. E, embryonic day; P, postnatal day; wks, weeks. (b) Same analysis as in a using poly(A)⁺ RNA (20 µg per slot) isolated from the indicated regions of adult male Sprague-Dawley rat brain. Medulla, medulla oblongata; hypo, hypothalamus; hippo, hippocampus; cortex, cerebral cortex; cblm, cerebellum; olf, olfactory bulb.

brain showed remarkable regional specificity with high levels in hippocampus compared with other brain regions analyzed (Fig. 2b). In fact, cerebellum was the only other region where HDNF mRNA was clearly detected, with the exception of

cerebral cortex, which showed a weak signal. BDNF mRNA was more widely distributed in rat brain, although hippocampus also contained the highest amount, followed by cerebral cortex, pons, and cerebellum (Fig. 2b).

Neurons Expressing HDNF and BDNF mRNA Are Located in a Distinct Topographical Arrangement in Hippocampus. Anterior sections of the dorsal hippocampus showed neurons expressing high levels of HDNF mRNA primarily confined to the medial part of CA1 and CA2 (Fig. 3a and c). Few HDNF mRNA-expressing neurons were also found in lateral parts of CA1. Granular cells of the dentate gyrus were also highly labeled (Fig. 3a). CA3 and hilar cells of the dentate gyrus showed no labeling for HDNF mRNA at any level (Fig. 3d). No labeling was seen over any sections after hybridization to a control probe, complementary to the specific HDNF probe. Adjacent sections hybridized to a BDNF-specific probe revealed labeling over granular neurons in the dentate gyrus (Fig. 3b), although possibly with lower intensity than that seen after hybridization for HDNF mRNA. Strong labeling with the BDNF-specific probe was found over neurons in the hilar region (Fig. 3e), CA3, and part of CA2 (Fig. 3b). Few BDNF mRNA-expressing neurons, which appeared to be less intensively labeled, were also detected in CA1 and CA2 (Fig. 3b). Intensely labeled neurons were seen in claustrum, located lateral to the external capsule. This region showed no labeling for HDNF mRNA.

Neurotrophic Activities of HDNF in Explanted Chicken Embryonic Ganglia. The 1020-bp HDNF cDNA insert was cloned in the expression vector pXM (34), designed for transient expression in COS cells. Two plasmid constructs were isolated, containing the HDNF insert either in the correct or opposite orientation for translation of the HDNF protein. The latter construct was used as a negative control. Included was also a construct containing the rat NGF gene (36). The different constructs were transfected into COS cells and 3 days later conditioned medium was tested for biological activity in bioassays that measured fiber outgrowth from various chicken embryo ganglia. A marked stimulation of neurite outgrowth, consistently resulting in circular or oval fiber halos, was seen in the ganglion of Remak, a ganglionated nerve trunk in the mesorectum of the chicken embryo (38, 39) (Fig. 4a). Although NGF is known to stimulate the explanted ganglion of Remak (39), it was far less efficient than HDNF (Fig. 4b). A modest stimulation of fiber outgrowth was also seen with HDNF in the nodose ganglion, consisting of neurons exclusively derived from an epidermal placode (22)

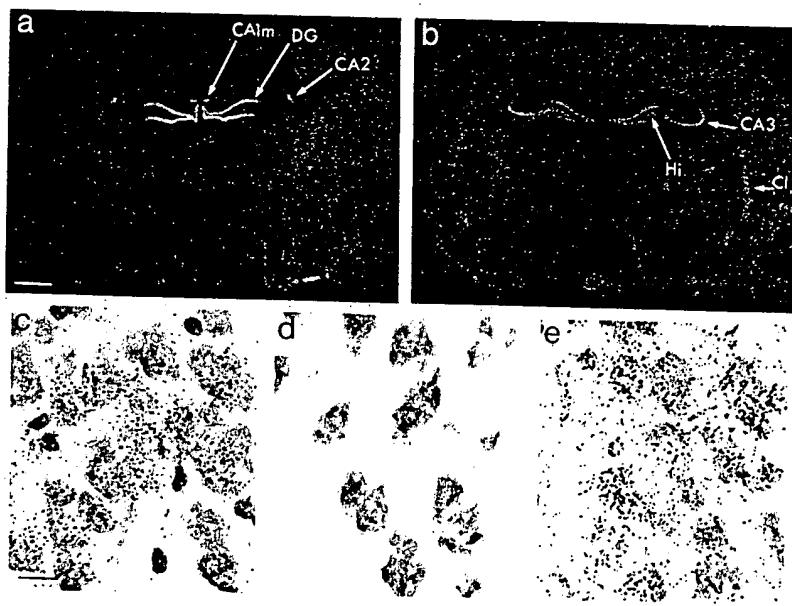


FIG. 3. Expression of HDNF and BDNF mRNA in hippocampal neurons. Rat (Sprague-Dawley) brain sections hybridized to either HDNF- or BDNF-specific oligonucleotide probes. (a) Autoradiogram from a section at the level of hippocampus hybridized to the HDNF-specific probe. Note labeling over medial CA1, CA2, and the dentate gyrus. (b) Adjacent section hybridized to a BDNF-specific probe. Note labeling over CA2 and CA3 as well as hilar cells and dentate granule layer. (c) Pyramidal neurons in medial CA1 labeled with the HDNF-specific probe. (d) Nonlabeled hilar neurons after hybridization to the HDNF-specific probe. (e) Hilar neurons labeled with the BDNF-specific probe. DG, dentate gyrus; CA1m, CA1 medial; Hi, hilus of dentate gyrus; Cl, claustrum. (a and b, bar = 1.3 mm; c–e, bar = 10 µm.)

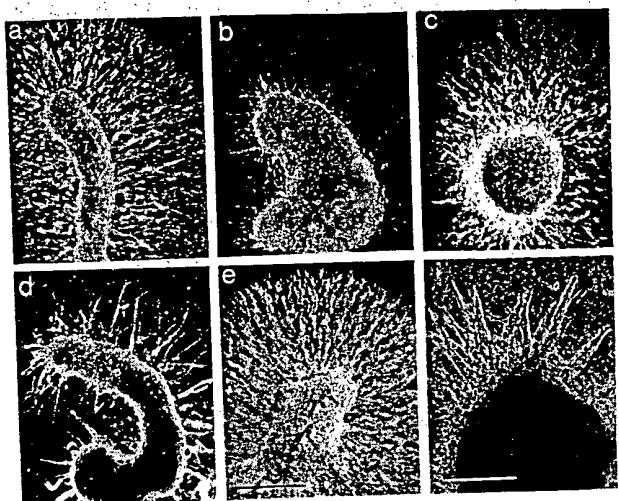


FIG. 4. Stimulation of fiber outgrowth from chicken embryonic ganglia. Biological activity of recombinant HDNF shown as effects on different nerve tissues from the chicken embryo. Remak ganglion stimulated by HDNF (a) or NGF (b). (c) Nodose ganglion with HDNF. Paravertebral sympathetic ganglion in response to HDNF (d) and recombinant rat NGF (e). (f) Ciliary ganglion with HDNF. All figures show ganglia after 1.5 days in culture. Dark-field microscopy. (Bars = 0.3 mm.)

(Fig. 4c). Again, HDNF was superior to NGF in evoking this response. A weak, but consistent, fiber outgrowth response with HDNF was seen in paravertebral sympathetic trunk ganglia (Fig. 4d), which, however, was much less pronounced compared with the massive response to rat NGF (Fig. 4e). In the ciliary ganglion, a weak but consistent fiber outgrowth response, manifested by the projection of short neurite fascicles, was seen with HDNF but never with NGF (Fig. 4c). In the dorsal root ganglia, HDNF stimulated neurite outgrowth to the same extent as NGF.

Displacement of NGF Binding to PC12 Cells by HDNF. Concentrated conditioned medium from transfected COS cells was tested for its ability to compete for binding ^{125}I -labeled NGF (^{125}I -NGF) to its receptor on PC12 cells. The concentration of ^{125}I -NGF used allowed $\approx 80\%$ of the labeled NGF to be bound to the low-affinity receptor site in the absence of competition (40). Twenty-five times concentrated medium containing the HDNF protein displaced $\approx 70\%$ of the labeled NGF and a 20% displacement was seen after a 25-fold dilution (Fig. 5). In contrast, 25 times concentrated medium from COS cells transfected with the HDNF cDNA in the opposite orientation did not show any displacement. Con-

centrated medium from cells transfected in parallel with a rat NGF gene displaced 50% of the labeled NGF when diluted 250 times.

DISCUSSION

The cDNA clone isolated in this study encodes a protein HDNF, with a remarkable sequence similarity to both NGF and BDNF and therefore represents an additional member of a family of neurotrophic proteins. Recently (at the time of submission of this manuscript), two groups (41, 42) independent of us isolated genomic clones for a protein (neurotrophin 3) from mouse and rat, respectively, which is identical to the neurotrophic protein characterized in this study. Our cDNA clone predicts a 282-amino-acid-long protein, which is 2 amino acids longer than the protein deduced from the genomic clones (41, 42). Two alternative start sites for translation of the NGF protein have been proposed; the first is located in a separate 5' exon (43). The second start site located in the 3' exon, is also efficiently used for translation of the NGF protein (36, 44) and generates a 68-amino acid shorter protein. Thus, the structure of our cDNA clone indicates that the HDNF protein utilizes two alternative start sites for translation, located in separate exons, and suggest that the genomic organization of HDNF and NGF is very similar.

In peripheral ganglia bioassays, HDNF showed neurotrophic activities that were to some extent reminiscent of both NGF and BDNF. Thus, in similarity to BDNF (20), HDNF stimulated fiber outgrowth from the nodose ganglion and, as for NGF, evoked a fiber outgrowth response in sympathetic ganglia. In the latter case, however, the response was clearly weaker than with NGF. The partially overlapping activities seen *in vitro* may reflect a cooperation of these factors *in vivo*, where two or more proteins from the same family may support the development and/or maintenance of specific neurons. The most striking stimulation of fiber outgrowth evoked by HDNF was seen in the peripheral autonomic ganglion of Remak containing mostly cholinergic but also some adrenergic neurons (38, 39). This effect was clearly more pronounced than effects seen with NGF (39) suggesting that HDNF also evokes trophic responses different from both NGF and BDNF. In agreement with this, HDNF showed a weak, but consistent, neurite outgrowth response in the ciliary ganglion, which does not respond to NGF or BDNF. The ciliary ganglion is known to respond to ciliary neurotrophic factor (45), which lacks a signal sequence, but could be released by an as yet unknown mechanism (46). Thus, HDNF is the only secreted neurotrophin today that is known to affect fiber outgrowth, at least *in vitro*, from the ciliary ganglion.

The HDNF protein displaced ^{125}I -NGF from PC12 cell indicating that it can interact with the NGF receptor. With the assumption that NGF and HDNF were produced in equal amounts in parallel transfections and that the conditioned medium lacks interfering substances, the interaction of NGF to its receptor was 30-fold more efficient. PC12 cells have both low- and high-affinity receptors but only the high affinity receptor mediates a biological response (47). The fact that recombinant rat NGF readily stimulated neurite outgrowth from PC12 cells, whereas HDNF, even at 30-fold higher concentrations than NGF, did not suggest that HDNF can only interact with the NGF receptor in a low-affinity form. It therefore appears likely that the biological responses elicited by HDNF are mediated by either a separate second messenger system compared with NGF or that the HDNF receptor is different from the NGF receptor.

In similarity with NGF, HDNF mRNA was found in several peripheral rat tissues, with the highest level in kidney. Hybridization of the same filters to a rat NGF probe revealed that the level of HDNF mRNA in kidney was only slight-

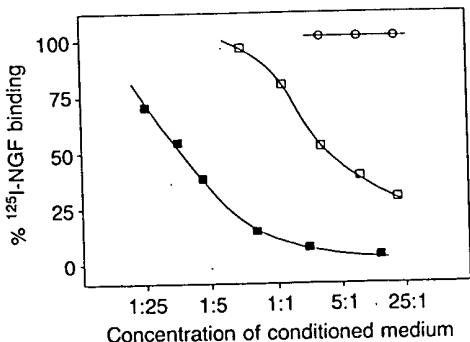


FIG. 5. Displacement of ^{125}I -NGF from its receptor on PC12 cells by HDNF and NGF. Serial dilutions of transfected COS cell medium with (□) or without (○) HDNF or containing rat NGF (■) were assayed for their ability to displace ^{125}I -NGF from its receptor on PC12 cells. Data are from two independent experiments that showed a variation of $\pm 20\%$.

higher than the levels of NGF mRNA in peripheral sympathetic target tissues, indicating that HDNF is produced in relatively small amounts in peripheral rat tissues. This is also true for the brain, and the fact that seven positive cDNA clones were isolated from 1.2×10^6 independent clones suggests that in hippocampus, containing the highest level of HDNF mRNA, this transcript constitutes ≈ 1 in every 170,000, which clearly represents a rare transcript. Thus, as in the case of NGF, HDNF may be present in limiting amounts and functions *in vivo* as a target-derived factor for a specific subset of both peripheral and central neurons. The regional distribution of HDNF mRNA in the periphery is, however, different from NGF, and, in agreement with the *in vitro* biological assays, HDNF may support a different set of peripheral neurons. Of interest is also that HDNF mRNA was found in the ovary, whereas no mRNA was detected in the testis, where both NGF and its receptor is expressed (48) and where NGF has been suggested to mediate an interaction between Sertoli cells and germ cells (49). This shows that different members of the NGF family are expressed in different reproductive tissues and suggests that they may have nonoverlapping functions outside the nervous system.

Interestingly, the three neurotrophic proteins were maximally expressed at different times of brain development with a peak of HDNF mRNA shortly after birth, BDNF mRNA around 2 weeks, and NGF mRNA around 3 weeks after birth (see ref. 8 for NGF). Moreover, the mRNA's for all three proteins were expressed in hippocampus at levels higher than in other regions, particularly in the case of HDNF. Within hippocampus, all three mRNAs were also confined to neurons (see ref. 10 for NGF) and a clear topographical division was seen, where HDNF mRNA was concentrated to pyramidal neurons in medial CA1, CA2, and granular neurons in dentate gyrus. Strongly labeled BDNF neurons were primarily seen in CA3 and the hilar region of dentate gyrus. Neurons with apparent lower levels of BDNF mRNA were seen in the dentate gyrus. The hilar region, containing neurons with high levels of BDNF mRNA, showed no labeling for HDNF mRNA.

This remarkable concentration of trophic factors in the adult hippocampus suggests that maintenance of plasticity is crucial to its function and may relate to the presumed morphological sequelae of long-term potentiation and memory consolidation processes. The intriguing temporal and spatial expression of the three neurotrophic proteins in the brain suggests that they predominantly support neuronal innervation at different times of development and that they may also exert specific trophic support for different central nervous system neurons, a possibility that will be an interesting topic for future studies.

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275.9

ACTIVATION OF SECOND MESSENGER PATHWAYS BY GDF-1.
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GDF-1 (growth/differentiation factor 1) is a member of the TGF- β superfamily that has been shown to be expressed primarily in the nervous system. GDF-1 is expressed as a 42 kd precursor protein which is processed to a 15 kDa mature form containing 7 cysteine residues with spacing and structural homology similar to TGF- β . We have been working towards identifying bioassays for GDF-1. Herein, we present the results of testing GDF-1 in various growth factor assays. Initial studies show that recombinant human GDF-1 stimulates the expression of the immediate early genes in neural cell lines. Further analysis of specific neural gene expression in primary hippocampal cells is included. Preliminary screens for potential receptor proteins and identification of potential second messenger pathways for GDF-1 will also be presented.

Society for Neuroscience Abstracts

275.11

NEUROTROPHIN-4/5 PROMOTES SURVIVAL OF DOPAMINERGIC NEURONS, AND IS PRESENT IN EMBRYONIC STRIATUM. M. Hynes, L. Berkemeier, K. Poulsen, F. Hefti*, and A. Rosenthal*. Department of Neuroscience Genentech, Inc. South San Francisco, California. 94080.

Parkinson's disease is a prevalent neurological disease characterized by profound and incapacitating movement disorders. A common pathology in Parkinson's patients is degeneration of substantia nigra dopaminergic neurons that innervate the striatum and a corresponding decrease in striatal dopamine content. We have investigated the ability of neurotrophin 4/5 (NT-4/5) to prevent or ameliorate degeneration of these dopaminergic neurons. Using an *in vitro* assay for dopaminergic survival we compared NT-4/5 to other neurotrophins, as well as to bFGF and CNTF. We find that NT-4/5 is as potent as brain-derived neurotrophic factor (BDNF) in promoting the survival of rat embryo mesencephalic dopaminergic neurons *in vitro* and that these factors were the most effective of those tested. However, large quantities ($1-2 \times 10^{-9}$ M) of either neurotrophin are required to produce an effect. Furthermore, we demonstrate that transcripts for NT-4/5 and BDNF are present in striatum and using double immunocytochemistry and *in situ* hybridization show that messenger RNA encoding full length trkB, a receptor for BDNF and NT4/5 is present on dopaminergic neurons in the substantia nigra of embryonic rats. In addition, both NT-4/5 and BDNF protect embryonic dopaminergic neurons from the toxic effects of the neurotoxin MPP+. Thus, BDNF and NT-4/5 could be physiological survival factors for midbrain dopaminergic neurons and may be useful as therapeutic agents for Parkinson's disease.

275.13

BASIC FIBROBLAST GROWTHFACTOR (bFGF) INCREASES DIVISION OF NEURONAL PRECURSORS IN SPHERES CONTAINING DOPAMINE NEURONS FROM SUBSTANTIA NIGRA PRIMORDIA OF RAT. M.M. Bouvier* and C. Mytilineou. Dept. Neurology, Mt. Sinai Sch. of Medicine, New York, N.Y., 10029.

Cultures were prepared from ventral mesencephalon of E12 rat embryos, a stage which coincides with the beginning of the birth of dopamine neurons. Cells were treated with bFGF (10ng/ml), EGF (10ng/ml), or both upon plating and daily thereafter. Cells were initially grown on a non-adhesive substrate, a process which prevents attachment and differentiation. Floating spheres of proliferating cells were cultured in this manner for 12 or 24 days *in vitro* (DIV), then replated on adhesive substrate where they attached and differentiated. 3 H-thymidine incorporation demonstrated a strong proliferative effect of bFGF at DIV 12, and neuronal division was demonstrated by double labelling with TAU and 5-bromo-2'-deoxyuridine (BrdU). All of these bFGF-treated spheres contained neurons and astrocytes at DIV 12, as demonstrated by staining with TAU and glial fibrillary acidic protein (GFAP), respectively. Many of these spheres contained dopamine neurons, as demonstrated by tyrosine hydroxylase (TH) staining. By DIV 24, the proliferative effect of bFGF had decreased, and virtually none of the spheres were TH positive. EGF had no proliferative effect on these cells at DIV 12, but had a strong proliferative effect by DIV 24. All of the EGF-treated spheres contained neurons and astrocytes, but virtually none contained dopamine neurons. In cultures treated with both growth factors, EGF was unable to modify the bFGF effect at DIV 12 or at DIV 24. Future experiments are envisioned to transplant the bFGF-treated spheres cultured for 12 days into 6-hydroxydopamine lesioned rats. Supported by The International Foundation of Parkinson Disease Association and The United Parkinson Foundation.

275.10

IGF-I INDUCED INCREASES IN DOPAMINERGIC CELL NUMBER AND/OR TYROSINE HYDROXYLASE APENZYME IN PRIMARY VENTRAL MESENCEPHALIC CULTURES ASSESSED BY A CELL-BASED ELISA. D. Bozyczko-Coyne, T. J. Connors, B. W. McKenna and N. T. Neff*. Cephalon, Inc., West Chester, PA 19380.

Dopaminergic (DA) neurons represent less than 1-2% of the total cell population of ventral mesencephalic (VM) cell cultures. These neurons, contributing to the DA nuclei of the substantia nigra, are among those compromised in Parkinson's disease. The discovery of factors or drugs that augment the survival/function of these neurons is impeded/limited by methods that require either direct counting of tyrosine hydroxylase (TH) positive cells or substantial numbers of cultured VM cells for assessment of TH enzyme activity. To circumvent these limitations, a 96 well format cell-based ELISA was developed for initial screening of factors with potential trophic activities. This assay measures VM culture TH apoprotein content. ELISA measurement of TH apoprotein content correlated directly with changes in the number of TH positive neurons. A linear assay range resulted from initial plating densities of $0.25 - 4 \times 10^3$ cells/well, representing a potential maximum of 500-8000 DA cells/well under basal culture conditions. Under these conditions, IGF-I consistently increased TH specific signal (1.5-2.0 fold) correlating to increased DA cell numbers. bFGF, EGF, NT-3 and BDNF were assessed by the TH cell-based ELISA for effect on TH specific signal in VM cultures. NT-3 and EGF did not elicit changes in VM culture TH apoprotein, while bFGF and BDNF additions resulted in small (20-40%) and variable increases in TH signal. Maximal increases in TH specific signal were observed when VM cells were cultured in the joint presence of IGF-I and EGF. Under these conditions, ELISA TH signals increased 2.5-3.0 fold over untreated control cultures representing a synergistic and/or cooperative effect of IGF-I and EGF on TH expression or DA neuronal survival.

275.12

'PURIFIED' RAT DOPAMINE NEURONS REQUIRE GROWTH FACTORS FOR THEIR SURVIVAL IN CULTURE. L.J. Lee, C.W. Kerr and L. Iacovitti*. Dept. of Neurology, Institute of Neuroscience, Hahnemann University School of Medicine, Philadelphia, PA 19102.

Dopamine neurons, because of their selective vulnerability in Parkinson's disease, have been the subject of intense investigation. Historically, those studies elucidating the mechanisms regulating dopamine cell survival have been done in tissue culture. A major obstacle encountered in these studies, however, has been the heterogeneity of cell types present; with dopamine neurons representing less than 0.1% of the total cellular composition. Therefore, in the present study, we have developed a method for isolating pure populations of dopamine neurons from the rat nigrostriatal system for the purposes of studying the role of growth factors in their survival. To do so, the fluorescent dye, Dil (1,1'-Diiodo-3,3'-tetramethylindocarbocyanine perchlorate; 2 μ l of 7 mg/ml) was injected into the striata of E16 rat embryos. During transport of the dye, the embryos were maintained in Avian Tyrode solution equilibrated with 95% O₂ and 5% CO₂ in a 31°C waterbath for 18-20 hours. The ventral midbrain of control and injected embryos were then microdissected and trypsin-dissociated. Cells were next passed through a Becton-Dickinson FACStar Plus cell sorter. The fluorescent* cells were plated into tissue culture wells and maintained either on defined media or on media supplemented with a cocktail of growth factors (aFGF, bFGF, EGF, CNTF, IGF, NGF, TGF β , IL1, and LIF). Two hours to 3 days later, cultures were fixed and examined either in the fluorescence microscope or were immunostained for the specific dopamine synthetic enzyme, tyrosine hydroxylase (TH). Greater than 90% of cells isolated in this manner exhibited fluorescence and TH immunolabeling immediately after plating. However, the survival of these neurons for 1 or more days in culture required the presence of trophic factors in the growth media. We conclude that nearly pure populations of dopamine neurons can be isolated by flow cytometry after Dil labeling. Moreover, dopamine neurons grown in the virtual absence of other cell types will not survive in culture without the addition of specific trophic factors to the growth media.

275.14

TROPHIC AND NEUROPROTECTIVE EFFECTS OF GLIAL CONDITIONED MEDIUM ON CULTURED MESENCEPHALIC DOPAMINE NEURONS. T.H. Park* and C. Mytilineou. Dept. Neurology, Mt. Sinai Sch. of Medicine, New York, N.Y., 10029.

Our previous studies demonstrated that astrocytic glia exerts trophic effects upon mesencephalic dopaminergic (DA) neurons in culture and protects against the toxicity produced by DA neurotoxins, 1-methyl-4-phenylpyridine (MPP $^+$) and 6-hydroxydopamine (6-OHDA). Since this protection may result from glial released factors in the extracellular medium, we examined whether or not conditioned medium (CM) from astrocytic cultures can mimic the trophic and protective effects produced by glia. Glial CM was obtained from enriched astrocytic cultures of mesencephalic or striatal origin, and then used as the maintenance medium for mesencephalic cells from embryonic day 14 rats grown on polyomithine substrate. Control cultures were maintained on serum-free chemically defined medium. 100 uM MPP $^+$ (60 min) or 50 uM 6-OHDA (45 min) was applied at day 6 *in vitro* and the extent of neurotoxicity was assessed 24 hrs later by 3 H-DA uptake and tyrosine hydroxylase (TH) immunocytochemistry. Both mesencephalic and striatal CM stimulated neurite outgrowth of DA neurons, resulting in increased uptake levels (3.8-fold and 2.8-fold increase over controls) and also promoted DA neuron survival (4.2 and 2.8-fold increase over controls). These trophic effects of CM on DA neurons are similar to those produced by glia. CM treatment also mimicked glial protective effects against MPP $^+$ and 6-OHDA induced reduction in DA uptake (from 88.7±3.7 to 59.2±8.0 % reduction for MPP $^+$ and from 60.3±2.9 to 23.8±5.5 % reduction for 6-OHDA). These data suggest that astrocytes secrete trophic substances into the medium, which can modify the sensitivity of DA neurons to neurotoxins. Supported by NIH grant NS-23017 and the American Parkinson Disease Association.

Distinct Modulatory Actions of TGF- β and LIF on Neurotrophin-Mediated Survival of Developing Sensory Neurons*

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The neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) are important for the regulation of survival and differentiation of distinct, largely non-overlapping populations of embryonic sensory neurons. We show here that the multifunctional cytokine transforming growth factor- β (TGF- β) fails to maintain sensory neurons cultured from embryonic day (E) 8 chick dorsal root ganglia (DRG), although DRG neurons are immunoreactive for the TGF- β receptor type II, which is essential for TGF- β signaling. However, in combination with various concentrations of NT-3 and NT-4, but not NGF, TGF- β causes a further significant increase in neuron survival. In DRG cell cultures treated with NGF, NT-3, and NT-4, a neutralizing antibody to TGF- β decreases neuron survival suggesting that endogenous TGF- β in these cultures affects the efficacies of neurotrophins. Consistent with this notion and a modulatory role of TGF- β in neurotrophin functions is the observation that TGF- β 2 and - β 3 immunoreactivities and TGF- β 3 mRNA are located in embryonic chick DRG in close association with neurons from E5 onwards. We also show that leukemia inhibitory factor (LIF) significantly decreases NGF-mediated DRG neuron survival. Together, these data indicate that actions and efficacies of neurotrophins are under distinct control by TGF- β and LIF in vitro, and possibly also in vivo.

KEY WORDS: Dorsal root ganglionic neurons; neurotrophic factors; nerve growth factor; brain-derived neurotrophic factor; neurotrophin-3; cell culture.

INTRODUCTION

Regulation of neuronal survival by neurotrophic factors is an essential aspect of nervous system development and injury. Current views concerning the functions and mechanisms of action of neurotrophic factors have largely been shaped by studies on nerve growth factor (NGF) and NGF-dependent sympathetic and sensory neurons (see 1,2 for reviews). However, the conceptually simple classical scenario, in which neurons

compete for limited amounts of a target-derived and retrogradely transported factor, specific for distinct types of neurons, fails to correctly describe the real complexity that exists in many central and peripheral neuronal systems. Such complexity results, e.g. from a high degree of molecular heterogeneity, pleiotropism, overlap in biological activities, multiplicity of sources, and distinct spatiotemporal regulation of expression of acting factors and their receptors (see 3).

There is increasing evidence indicative of potential interactions between different classes of factors in the regulation of neuron survival. Thus NGF, and the "neurotrophic cytokines", leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF), have been re-

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ported to be equally effective in promoting the survival of dorsal root ganglionic (DRG) neurons of neonatal rodents (4,5). NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and an unidentified non-neurotrophin from chromaffin granules promote the survival of DRG neurons cultured from embryonic day (E) 8 chick embryos in a partly overlapping fashion (6,7). Another important issue related to the interactions of different factors is how neuronal sensitivity to neurotrophins is regulated and how neurotrophin expression is controlled. Several studies have shown that, e.g. neural activity (8,9) as well as the cytokines interleukin-1 (IL-1; 10) and transforming growth factor- β (TGF- β ; 11-13) can influence neurotrophin expression, and that neurotrophin receptor expression may be affected by neurotrophins themselves (14).

In the present study we show that TGF- β 2 and - β 3 immunoreactivities and TGF- β 3 mRNA can be detected in embryonic chick DRG. DRG neurons isolated at E8 are immunoreactive for the TGF- β receptor type II (T β RII), but do not respond in vitro to TGF- β treatment with increased survival. However, TGF- β potentiates survival achieved with a wide range of concentrations of NT-3 and NT-4, while LIF significantly reduces NGF-mediated survival of DRG neurons. These data suggest that TGF- β and LIF can control DRG neuron sensitivity to neurotrophins.

EXPERIMENTAL PROCEDURE

Growth Factors. Growth factors were purchased from Boehringer, Mannheim (NGF, 2.5 S NGF; recombinant human (rh) LIF), R & D Systems (rhTGF- β 1, - β 2, - β 3), and IC Chemikalien, Ismaning, Germany (rhNT-3, rhNT-4). Lyophilized factors were resuspended in culture medium (see below), to give a final concentration of 1 μ g/ml and stored in aliquots of 100 μ l at -70°C until use.

Tissue Culture. Fertilized white Leghorn chick eggs were purchased from a local aviary and incubated in a humidified egg chamber at 38°C until E8. Lumbar DRG were removed, freed from nerve roots and connective tissue and collected in ice-cold Ca^{2+} , Mg^{2+} -free Hanks' Balanced Salt Solution (CMF). Following incubation in 0.08% trypsin (ICN, 15 min), ganglia were washed in culture medium (see below) and dissociated using fire-polished Pasteur pipettes. Cell suspensions (neuron/non-neuron ratio 1:2) were plated at 1,200 cells per well in 50 μ l culture medium in 96-well microtiter plates (Costar A/2) precoated with polyornithine and laminin as described (15). Culture medium was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with the N1 additives (16), 0.25% BSA and 100 U/ml penicillin. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 /95% air. After 48 hours cultures were fixed by addition of 2.5% glutaraldehyde in phosphate-buffered saline (PBS). Numbers of surviving neurons were determined by direct counting of 30% of the surface area using phase contrast microscopy.

Immunocytochemistry. White Leghorn chick embryos (day 3/stage 18 to day 15) were fixed by immersion or perfusion via the

heart followed by immersion in ice-cold 4% phosphate-buffered neutral formalin. After 24 hours this solution was replaced by ice-cold PBS, PBS/50% ethanol, and tissues were dehydrated through a graded series of ethanol and embedded in paraffin wax. Isolated DRG neurons from E8 chick embryos were seeded on polyornithine/laminin-coated glass coverslips at a density of 80 neurons per mm² and maintained for various periods of time with or without NGF (10 ng/ml) as described above. Cells were fixed in neutral formalin.

Antibodies against TGF- β 2 and TGF- β 3 (generously supplied by Dr. K. Flanders, NCI, NIH) and T β RII (UBI) have previously been characterized and described in detail (17-19). In brief, the antibody against TGF- β 2 is directed against peptide sequence 50-75 of mature TGF- β 2. Anti-TGF- β 3 antibodies are directed against amino acids 50-60 of the mature regions or amino acids 81-100 in the pro-region of the pre-pro-TGF- β 3. Antibodies to TGF- β 3 gave identical staining patterns. The antibody to the T β RII recognized a single band at approximately 66 kDa in Western blots of E8 chick DRG homogenates corresponding to the correct molecular mass of the chick T β RII (20). Antibodies were incubated overnight at 4°C and visualized either using the Vectastain ABC-kit (anti-TGF- β s) or indirect immunofluorescence using TRITC-conjugated anti-rabbit IgG (anti-T β RII) (13).

In Situ Hybridization. A TGF- β 3-specific probe comprising a 732 nucleotide fragment spanning amino acid residues 8 to 251 of the precursor polypeptide was oriented in pBluescript KSII (Stratagene) such that the antisense probe was generated using the T3 promoter (21). "Sense" and "antisense" RNA probes were alpha ³²S-UTP-labeled (1200 Ci mmol⁻¹, New England Nuclear) to a specific activity of >10⁹ disintegrations min⁻¹ μ g⁻¹ using the appropriate T3 and T7 transcription system. The probe was digested to an average length of 100 nt by controlled alkaline hydrolysis and used at a final concentration of 40 pg μ l⁻¹ (4 \times 10⁴ disintegrations min⁻¹ μ l⁻¹). Five to seven μ m thick sections, placed on TESPA (Fluka 109324) coated slides, were baked overnight at 42°C, deparaffinized, rehydrated, and refixed in 4% paraformaldehyde/PBS. They were then treated as described in detail (13).

Statistical Analysis. Data were analyzed by a one-way ANOVA, and the significance of intergroup differences were determined by applying Student's t-test. Differences were considered significant at *P < 0.05, **P < 0.01, ***P < 0.001.

RESULTS

TGF- β 2 and - β 3 Immunoreactivities and TGF- β 3 mRNA in Developing DRG. TGF- β 2 and - β 3 are the prominent, strictly co-localizing TGF- β isoforms in the developing and adult rodent and chick nervous system (13,22,23). Fig. 1 illustrates the development of TGF- β 3 immunoreactivities (ir) in chick DRG *in situ* between E5 (Fig. 1A) and E15 (Fig. 1C). At E5 (Fig. 1A) and E9 (Fig. 1B), immunopositive fiber-like structures surround neurons, which appear mostly immunonegative. At the light microscopic level it is not possible to definitely assign the TGF- β ir to satellite cells or extracellular matrix, or to exclude that neurons have TGF- β located in the subplasmalemmal region. At E15 (Fig. 1C) some, but not all DRG neuronal perikarya are TGF- β 3-ir. *In situ* hybridization revealed specific expression of TGF- β 3 mRNA in E11 DRG (Fig. 1D). Labeling was

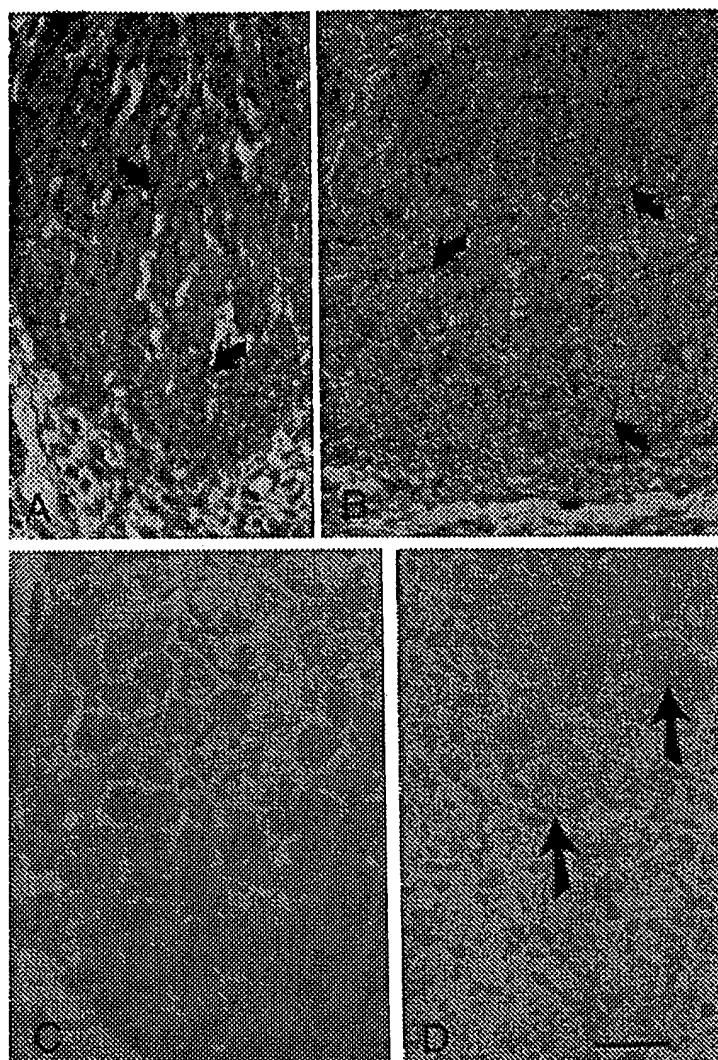


Fig. 1. TGF- β 3 immunoreactivity (A, B, C) and mRNA (D) in dorsal root ganglia from E5 (A), E9 (B), E15 (C) and E11 (D) chick embryos. At E5 and E9 TGF- β 3 immunoreactivity (arrows) surrounds developing neurons and may be located in satellite cells or extracellular matrix. Radioautographic *in situ* hybridization (D) suggests that satellite cells may be one site of synthesis of TGF- β 3 during DRG development. In older DRG (E15, C) TGF- β 3 immunoreactivity is clearly detectable in neuronal cell bodies. Bar = 25 μ m in D, = 50 μ m in A-C.

most intense peripherally to neuronal cell bodies suggesting localization in satellite cells.

Presence and Localization of T β RII in DRG Neurons. T β RII is the receptor component in the heteromeric type I/type II TGF- β receptor complex, which is essential for TGF- β transmembrane signaling (24). For demonstrating the presence of the T β RII, DRG neurons were isolated from E8 chick ganglia and maintained on coverslips 6 or 48 hours in the absence or presence of NGF (10 ng/ml). Prior to 6 hours neurons were not sufficiently adhesive for immunocytochemical processing. As shown in Fig. 2, all DRG neurons were immuno-

positive. The signal was specific in that it was abolished by absorbing the antibody to a DRG homogenate (not shown). Western blots of E8 chick DRG homogenates employing the same antibody revealed a single band at approximately 66 kDa (not shown), the relative molecular mass of the recently cloned chick T β RII (20).

Modulation of Neurotrophin-Mediated Neuronal Survival by Exogenous TGF- β . Fig. 3 shows the survival promoting capacity of saturating concentrations of NGF (10 ng/ml), NT-3 (10 ng/ml), and NT-4 (25 ng/ml; used instead of BDNF as a ligand for the trkB receptor) for DRG neurons in cultures maintained for 48 hours. TGF-

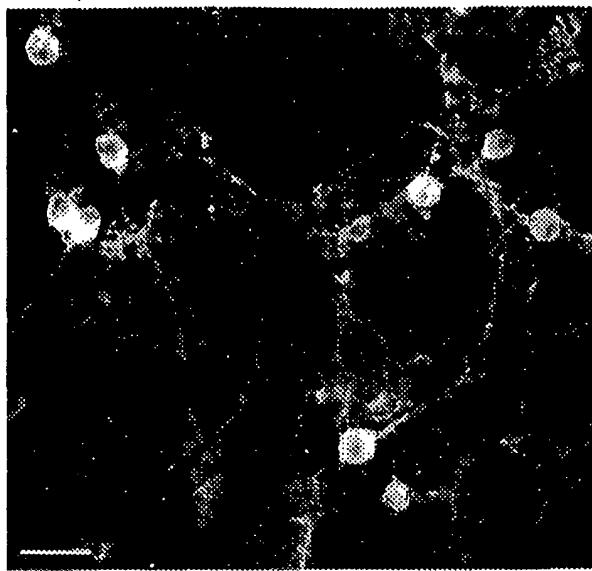


Fig. 2. T β RII immunoreactivity in DRG neurons cultured from E8 chick embryos. Cells were treated with NGF (10 ng/ml) for 24 hours and then processed for T β RII immunoreactivity visualized by indirect immunofluorescence using TRITC-conjugated anti-rabbit antibodies. Both neuronal cell bodies and processes are reactive for T β RII. Bar = 50 μ m.

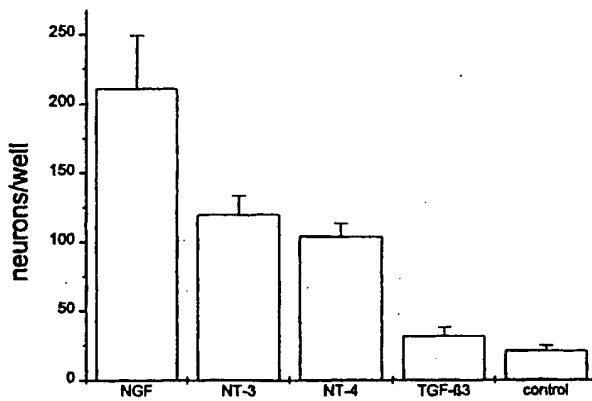


Fig. 3. Survival of E8 DRG neurons maintained for 48 hours in the presence of saturating concentrations of NGF, NT-3, NT-4, TGF- β , or without exogenous factors. Values given are means \pm SEM of triplicate determinations of at least two independent experiments.

β 3 (10 ng/ml) marginally enhanced neuron survival in the absence of other exogenous factors to approximately 15% of the NGF plateau. However, when a constant amount of TGF- β 3 (1.25 or 10 ng/ml) was combined with a wide range of different concentrations of NT-3 or NT-4, it significantly elevated plateau survival (Fig. 4A, B). Neuron numbers were increased approximately 1.6-fold when treated with TGF- β 3 plus NT-4 or NT-3, respectively, as compared to each neurotrophin alone.

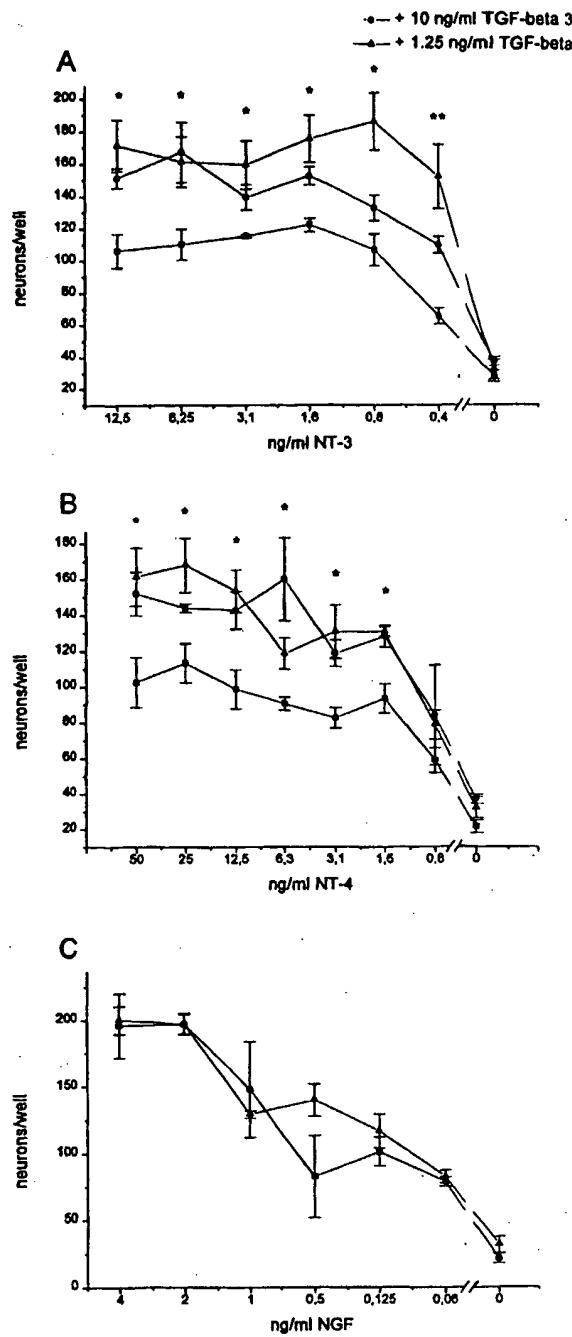


Fig. 4. Co-treatment of E8 DRG neurons for 48 hours with TGF- β 3 (at a constant amount of 10 and 1.25 ng/ml, respectively) and a broad range of different concentrations of NT-3 (A), NT-4 (B), and NGF (C). ■ refers to neurotrophin treatment in the absence of TGF- β . Surviving neurons were counted in triplicate determinations of at least two independent experiments. Values are given as means \pm SEM. Significance was derived from comparison between the experimental variants and the value for the growth factor alone. *P < 0.05, **P < 0.01.

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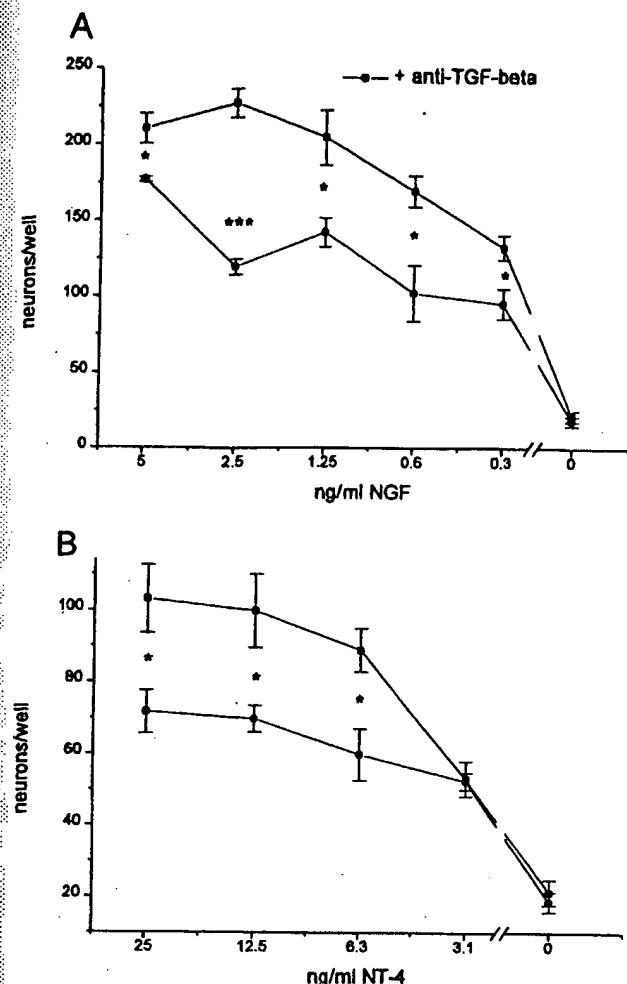


Fig. 5. Neutralizing antibodies to TGF- β 1/2/3 added to NGF-(A) and NT-4-(B) treated cultures of E8 DRG neurons reduce neuronal survival. ■ refers to neurotrophin treatment in the absence of antibodies to TGF- β . Values given are means \pm SEM of triplicate determinations of at least two independent experiments. Values are given as means \pm SEM. Significance was derived from comparison between the experimental variant and the value for the growth factor alone. *P < 0.05, ***P < 0.001.

The magnitude of this combined effect was greater than the calculated sum of the survival promoting effect of the individual factors. TGF- β 3, however, did not add to the survival promoting effect of NGF (Fig. 4C).

Modulation of Neurotrophin-Mediated Neuronal Survival by Endogenous TGF- β . We next addressed the issue whether TGF- β endogenous to the cultures might synergistically influence neurotrophin-mediated survival of DRG neurons. Cultures were run in the absence or presence of a wide range of concentrations of NGF, NT-3, or NT-4, with or without adding neutralizing antibodies to TGF- β 1/2/3 (10 μ ml). Amounts of anti-TGF- β 1/2/3

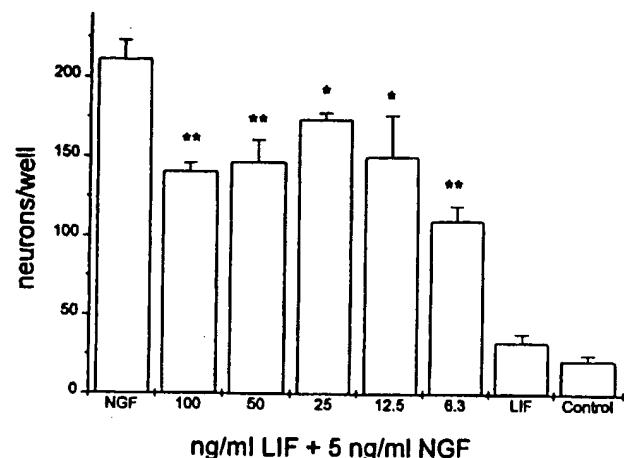


Fig. 6. Survival of E8 DRG neurons cultured for 48 hours and treated with NGF, LIF, or a combination of 5 ng/ml NGF and different concentrations of LIF. While LIF by itself has a small survival promoting effect, it significantly reduces NGF-mediated neuron survival. Values given are means \pm SEM of triplicate determinations of at least two independent experiments. Values are given as means \pm SEM. Significance was derived from comparison between the experimental variant and the value for the growth factor alone. *P < 0.05, **P < 0.01.

added were sufficient to reduce the biological activity of 3 ng/ml of TGF- β determined in an assay using mink lung epithelial cells (MLEC) by at least 95%, and the activity of 10 ng/ml TGF- β by at least 75% (cf. 25; not shown). Fig. 5 shows that anti-TGF- β antibodies significantly reduced neuronal survival in the presence of NGF, NT-3 (not shown), and NT-4 suggesting that TGF- β in these cultures in fact modulated DRG neuron survival.

Modulation of Neurotrophin-Mediated Neuronal Survival by LIF. LIF, a pleiotrophic cytokine, initially characterized by its ability to act on certain neurons as a transmitter determination factor (26), has been shown to affect the development and survival of cultured sensory (27,28) and spinal cord motor neurons (29,30). When LIF was added to E8 DRG cultures at 10 ng/ml (Fig. 6), neuronal survival was marginally enhanced (17% of NGF plateau). Concentrations lower or higher than 10 ng/ml were equally uneffective (not shown). In combination with a saturating concentration of NGF (10 ng/ml), however, LIF significantly reduced neuron survival (Fig. 6).

DISCUSSION

The NGF paradigm has long been and still is a strong conceptual basis for understanding the regulation of neuronal survival by neurotrophic factors (see 1). The simplest version of the neurotrophic factor concept im-

plies that trophic factor quantity is the essential survival limiting value, and that alterations in neuronal survival and death may be achieved by up- or downregulation of factor synthesis and release by target cells. Multiple evidence suggests that the neurotrophic scenario is far more complex than previously thought (see 3). Revisions in the neurotrophic factor concept must take into account, *inter alia*, (i) anterograde, para- and autocrine pathways of delivery of trophic molecules, (ii) convergent actions of multiple factors upon a single neuron, (iii) switches in factor dependence during development, (iv) existence of molecules actively promoting cell death, and (v) existence of molecules changing the sensitivity of neurons to a given set of trophic factors.

The present study has addressed the role of TGF- β and LIF in the regulation of survival of chick DRG neurons. TGF- β occurs in at least two isoforms, 2 and 3, within developing chick (13 and this study) and mouse DRG (22). Moreover, adult rat DRG neurons have been shown to synthesize and release within hours after injury TGF- β 1 (31). Consistent with the documented localization and upregulation and a presumed role of TGF- β as a modulator of neuron survival, our data provide the first evidence that TGF- β is a potent modulator in the NT-3 and NT-4-mediated survival response of DRG neurons. TGF- β , when added exogenously, significantly enhanced the effects of these neurotrophins. Mechanisms underlying the synergism of neurotrophins and TGF- β remain to be explored, but may be sought in several directions, including enhanced presentation of neurotrophins to their receptor systems, alterations in trkB/trkC/p75 expression or configuration, induction of neurotrophin synthesis in the cultures, or effects on signal transduction pathways distal to receptors.

TGF- β has been shown to be a strong inducer of NGF mRNA in cultured-astrocytes (11,32) and embryonic cutaneous cells (12), but not in Schwann cells (isolated from rat sciatic nerve; 33). The possibility that TGF- β might have increased NGF synthesis in our cultures has not been tested. It is conceivable that the slight elevation of neuron survival brought about by TGF- β resulted from the generation of a small amount of NGF by embryonic ganglionic satellite cells, which might differ from peripheral nerve Schwann cells (cf. 33) in their capacity for NGF synthesis.

It is not known and has not been addressed in this study, whether TGF- β can induce expression of trk receptors. If so, synergistic effects of TGF- β and NT-3/NT-4 might result from induction of trkC/trkB receptors on neuron populations, which constitutively lack the respective receptors.

Synergistic and coordinated trophic actions of multiple growth factors from different gene families upon neurons are gradually becoming acknowledged as an important means for fine tuning of the trophic support of neurons. The fact that combinations of factors act synergistically *in vitro*, and possibly also *in vivo*, has so far best exemplified on motoneurons (cf. 34). To our knowledge, there is only one case so far, however, suggesting that a factor, which by itself is inactive, can boost the efficacy of another trophic factor: on cultured motoneurons FGF-2 has a modest survival promoting effect unless neurons are primed with serum (35) or co-treated with TGF- β (Henderson, personal communication).

The role of TGF- β in exerting the effects described in the present paper, are not too surprising in the light of multiple evidence documenting the contextual and pleiotropic actions of TGF- β on non-neuronal cells (cf. 36, for review). For example, synergistic interaction of FGF-2 and TGF- β is required for promoting early embryonic development (37), induction of chondrogenesis (38), stimulation of Schwann cell proliferation (39), and inhibition of astroglial cell division (40). Our present data illuminate TGF- β as an important modulator for neurotrophin actions. Future studies will have to unravel the molecular bases of this modulatory function.

With regard to LIF, member of an unusual family of proteins termed the hematopoietic cytokines (see 41, for review), our data suggest a capacity to down-regulate the responsiveness of DRG neurons to NGF. This is reminiscent of similar actions of LIF and CNTF on cultured sympathetic neurons, where both cytokines induce programmed cell death (42) and CNTF has been shown to down-regulate the responsiveness to NGF (43). How these and our data can be reconciled with the potential of LIF to rescue axotomized DRG and motoneurons from cell death (28,44) is unclear at present. Possibly, enhanced regeneration of axons in a transected sciatic nerve of LIF-deficient mice (Patterson, personal communication) might be consistent with the notion that LIF can have adverse effects on neuron survival under yet to be defined particular conditions.

ACKNOWLEDGMENTS

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THE TRANSFORMING GROWTH FACTOR- β FAMILY

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KEY WORDS: growth inhibitors/suppressors, cell differentiation, cell adhesion, growth factor receptors, transformations

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INTRODUCTION

Secretory polypeptides are broadly used as mediators of intercellular communication to guide tissue development in metazoa. A decade ago, as the characterization of various mitogenic polypeptides and the isolation of scores of new ones were in progress, searches were launched to identify novel factors with activities other than promotion of cell growth. Some of the fruitful searches led to the isolation of a promoter of a transformed phenotype in fibroblasts, an inhibitor of cell proliferation, an inducer of chondrogenic differentiation, an inhibitor of myogenic differentiation, and an immunosuppressor. It was a major surprise to find that a single factor was responsible for all of these activities. Transforming growth factor type- β , or TGF- β , became the conventional name for this multifunctional factor, even though this name misrepresents the fact that TGF- β does not cause oncogenic transformation.

Besides being multifunctional, TGF- β represents a large family of factors with diverse activities. The concept that TGF- β is prototypic of a superfamily of growth, differentiation, and morphogenesis factors became clear in 1987 (Massagué 1987; Sporn et al 1987) following the rich harvest that yielded the inhibins, activins, Müllerian inhibiting substance, decapentaplegic product, and TGF- β 2. One after another, these factors proved to be structurally related to TGF- β . This family now includes embryogenic morphogens, regulators of endocrine function, and broad-spectrum as well as specialized regulators of cell proliferation and differentiation. The distribution of TGF- β -related factors is widespread in organisms from fruit flies to humans, and their evolutionary conservation is unusually strict. These factors appear to be involved in many processes of tissue development and repair.

We have learned much about the structure, expression, and activity of the TGF- β -related factors, and their implications in physiology, pathology, and therapeutics. Some glimpses of their receptors and mechanisms of action have been caught too. Herein I will attempt to appraise the current status of the studies of the TGF- β family and point out some directions, challenges, and opportunities for the future.

THE TGF- β SUPERFAMILY

Prototype Structure

The structural prototype for this gene superfamily is the protein that was first isolated from human platelets as TGF- β (Assoian et al 1983), cloned from a human cDNA library (Deryck et al 1985), and later named TGF-

β 1 (Chen et al 1985). It consists of 112 amino acid residues and contains a domain of 100 residues that is characteristic of a secretory translocator (Deryck et al 1985). This site is a secretory bioactive domain.

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β 1 (Cheifetz et al 1987). TGF- β 1 is a disulfide-linked dimer of two identical chains of 112 amino acids. Each chain is synthesized as the C-terminal domain of a 390 amino acid precursor that has the characteristics of a secretory polypeptide; it contains a hydrophobic signal sequence for translocation across the endoplasmic reticulum and is glycosylated (Deryck et al 1985; Purchio et al 1988) (Figure 1). The precursor cleavage site is a sequence of four basic amino acids immediately preceding the bioactive domain.

This precursor structure is shared by all known members of the superfamily with the exception of the TGF- β 4 precursor, which lacks a dis-

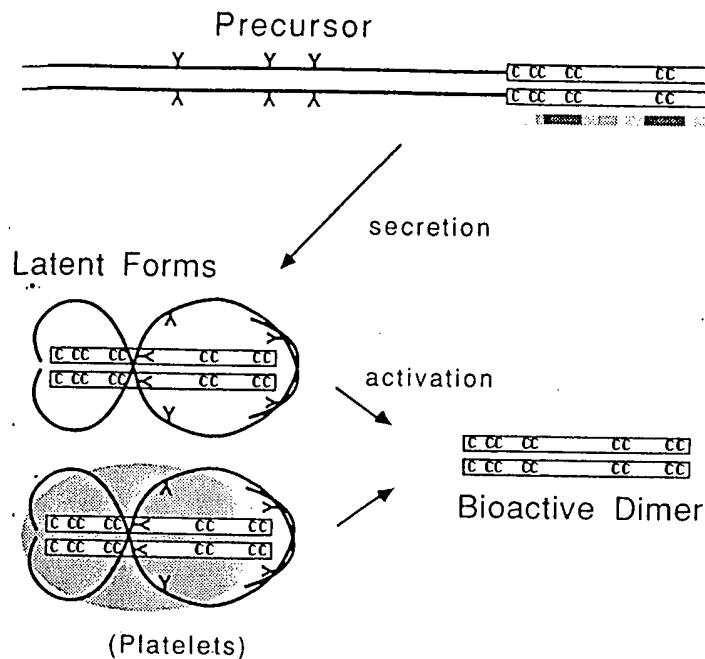


Figure 1 Precursor, latent, and bioactive forms of TGF- β 1. The TGF- β 1 precursor consists of an N-terminal signal sequence (thin line), a pro-region (thick line) and the C-terminal bioactive domain (box). The approximate locations of the three N-linked glycosylation sites (Y) in the pro-region and the 9 cysteines (C) in the bioactive domain are indicated. The intensity of the shadowing underlining the bioactive domain indicates the degree of amino acid sequence conservation throughout this domain in the other members of the TGF- β superfamily. After secretion, the cleaved pro-region remains associated with the TGF- β 1 dimer forming a biologically latent complex. In platelets and certain cell lines, the latent complex also contains a 125-190-kd glycoprotein of unknown function (shadowed). Bioactive TGF- β 1 is released by disassembly of this complex.

cernable signal sequence (Jakowlew et al 1988b). The known TGF- β -related factors can be grouped in four families according to their degree of structural or functional relationship (Table 1). Most of the amino acid sequence similarity between family members is in the C-terminal domain of the precursor. The degree of amino acid sequence identity in this domain ranges from 25 to 90% between different family members. The regions with highest homology are indicated in Figure 1. At least seven of the cysteines in this domain are conserved in all members of the superfamily, and all nine cysteines are conserved in the TGFs- β and the inhibin β chains. Except in Müllerian inhibiting substance (MIS) (Cate et al 1986), this domain is cleaved to generate a mature polypeptide similar in size to

Table 1 The TGF- β gene superfamily

Gene	Chromosome		Bioactive dimers	
	human	mouse	name	composition
TGF-β Family				
TGF- $\beta 1$	19q13	7	TGF- $\beta 1$	homodimer
TGF- $\beta 2$	1q41	1	TGF- $\beta 2$	homodimer
TGF- $\beta 3$	14q24	2	TGF- $\beta 3$	homodimer
TGF- $\beta 4^a$			(cDNA only)	
TGF- $\beta 5^b$			TGF- $\beta 5$	homodimer
			TGF- $\beta 1.2$	heterodimer
Inhibin Family				
α			Inhibin A	$\alpha.\beta A$ dimer
βA			Inhibin B	$\alpha.\beta B$ dimer
βB			Activin A	βA homodimer
			Activin AB	$\beta A.\beta B$ dimer
DPP/VG1 Family				
DPP-C ^c			(cDNA only)	
Vg1 ^b			(cDNA only)	
vgr-1			(cDNA only)	
BMP-2	13		BMP-2 homodimer	
BMP-3	2		homo or heterodimers	
BMP-4	5		(cDNA only)	
BMP-5	14,X		(cDNA only)	
BMP-6			homo or heterodimers	
BMP-7			homo or heterodimers	
Müllerian Inhibiting Substance Family				
MIS	19		MIS	homodimer

^a, ^b, and ^c are from chick, *Xenopus*, and *Drosophila*, respectively. Chromosomal locations are from Dickinson et al (1990), Fujii et al (1986), Barton et al (1988) and ten Dijke et al (1988b).

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mature TGF- β 1. Most of the factors in the family have been isolated as dimers from natural or recombinant sources.

The N-terminal pro-region of a given factor may be conserved between animal species but is usually divergent between different factors (Derynck et al 1986; Cate et al 1986). Two potential functions of the pro-region are to assist in the folding of the bioactive domain during synthesis and, at least in the case of TGF- β 1, to bind the mature factor forming a latent complex (see below).

TGFs- β : Forms, Expression, and Regulation

FORMS TGF- β is a term that refers to the dimeric products of various genes, five to date, identified by isolation of the proteins or by cDNA cloning. TGF- β was initially described as an activity produced by retrovirally-transformed cells (Roberts et al 1981), but it is now clear that TGFs- β are expressed in many normal cells and tissues and that this expression is not a unique attribute of the transformed phenotype. TGF- β 1 has been purified from human and porcine blood platelets (Assoian et al 1983), which are the richest source of TGF- β 1 (20 mg/kg), from human placenta (Frolik et al 1983), and bovine kidney (Roberts et al 1983).

Porcine platelets (Cheifetz et al 1987) and bovine bone (Seyedin et al 1987) yield TGF- β 2 in addition to TGF- β 1. TGF- β 1 and 2 were identified in bone based on their cartilage-inducing activity, and they were named CIF-A and CIF-B before their identity with TGF- β 1 and 2 was known (Seyedin et al 1985). TGF- β 2 was also independently discovered by its activity as a growth inhibitor (Holley et al 1980) or as an immuno-suppressor (Wrann et al 1987). TGF- β 2 cDNAs have been cloned from human, monkey, and mouse libraries (de Martin et al 1987; Madisen et al 1988; Hanks et al 1988; Miller et al 1989a).

Human TGF- β 3 was identified first at the cDNA level (ten Dijke et al 1988a; Derynck et al 1988) and was subsequently expressed in recombinant form (Graycar et al 1989; ten Dijke et al 1990). A chick embryo chondrocyte cDNA library yielded cDNAs corresponding to TGF- β 3 and TGF- β 4 (Jakowlew et al 1988a,b). TGF- β 5 was identified as a cDNA from *Xenopus laevis* (Kondaiah et al 1990) and has been purified from *Xenopus* XTC cell cultures (Roberts et al 1989). Mammalian TGF- β 4 and 5 have not been described yet. The complexity of this family may be greatly amplified by the existence of additional members and by the formation of heterodimers between different TGF- β gene products co-expressed in the same cell. The existence of the TGF- β 1/TGF- β 2 heterodimer (TGF- β 1.2) has been confirmed in porcine platelets (Cheifetz et al 1987).

STRUCTURAL CONSERVATION The degree of identity between the five

mature TGF- β sequences ranges from 64% (TGF- β 1 vs TGF- β 4) to 82% (TGF- β 2 vs TGF- β 4) (Kondaiah et al 1990), but individually TGFs- β are extremely well conserved. Thus there is > 97% identity between the mature TGF- β 1 sequences from various mammalian and avian species (Derynck et al 1987; Jakowlew et al 1988c), and the same is true for TGF- β 2 and 3 (Madisen et al 1988; Jakowlew et al 1988a). Conservation is also evident at the genomic level. The TGF- β 1 gene in various mammalian species has a seven-exon structure (Derynck et al 1987; Van Obberghen-Schilling et al 1987) that is largely conserved in other TGF- β genes (Derynck et al 1988). This conservation suggests that the TGFs- β arose by duplication of a common ancestor. The various TGF- β genes, however, are located in separate chromosomes in both man and mouse (Table 1).

Multiplicity of TGF- β forms and sequence conservation within each form through evolution suggest important specific roles for each of the TGFs- β . Differences are manifested in the pattern of expression of the various TGFs- β in vivo (see below) and in their ability to interact with different cell surface receptors (Cheifetz et al 1987). Acting on cultured cells, TGF- β 1, 2, and 3 often display similar activity and potency (Cheifetz et al 1987; Seyedin et al 1987; Graycar et al 1989), but show marked differences in certain cases (Ohta et al 1987; Ottmann & Pelus 1988; Tsunawaki et al 1988; Jennings et al 1988; Cheifetz et al 1990). Differences between the activity of TGF- β 1 and 2 have also been noted in vivo (Rosa et al 1988). The high degree of conservation of the individual TGF- β sequences suggests the existence of evolutionary pressure to retain certain specific features of each of these factors. Such features should become apparent from a better characterization of their individual activities and the resolution of their three-dimensional structures.

EXPRESSION PATTERNS Numerous cell types in culture express one or multiple forms of TGF- β , at least at the mRNA level (Derynck et al 1988). In general, the pattern of expression of the different TGFs- β varies with each cell type and does not appear to be uniform among cells of the same lineage. Expression of TGF- β is active throughout embryonic development and into adulthood (Heine et al 1987; Rappolee et al 1988; Thompson et al 1989; Miller et al 1989a). Histochemical localization studies have shown expression of TGF- β 1 and TGF- β 2 mRNAs or proteins in discrete regions of many tissues with characteristic temporal patterns. In the mouse embryo, TGF- β 1 mRNA is detectable in lung, intestine, and kidney mesenchymes, epithelial structures, megakaryocytes, osteocytes, and centers of hematopoiesis (Lehnert & Akhurst 1988; Wilcox & Derynck 1988). TGF- β 2 mRNA is detectable in gastrointestinal and tracheal submucosae, blood vessels, skin, cartilage, and bone (Pelton et al 1989). TGF- β

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immunostaining is high in mesodermal structures including teeth, larynx, palate, heart valves, cartilages, bones, and hair follicles (Heine et al 1987). At least 12 tissues and organs from adult mouse show expression of mRNAs for TGF- β 1, TGF- β 2, and/or TGF- β 3 (Thompson et al 1989; Miller et al 1989a,b); TGF- β 1 immunoreactivity is present in cells of the adrenal cortex, bone marrow, cardiac myocytes, chondrocytes, renal distal tubules, ovarian glandular cells, and chorionic cells of the placenta, and in extracellular matrices of the cartilage, skin, heart, pancreas, placenta, and uterus (Thompson et al 1989). The immunohistological distribution of a particular TGF- β form does not always match the distribution of the corresponding mRNA, a discrepancy that could result from diffusion and accumulation of the protein away from the sites of synthesis, a lack of translation of the mRNA (Assoian et al 1987), or immune cross-reactivity with other forms of TGF- β .

CONTROL OF ACTIVITY The existence of mechanisms that tightly control the expression and activity of TGF- β may be expected because numerous cell types express and can respond to these factors. TGF- β expression and activity are controlled by (a) regulation of TGF- β gene transcription, (b) production of TGF- β as a latent factor, and (c) sequestration of activated TGFs- β by extracellular matrix and circulating proteins.

Transcription of the TGF- β 1 gene can be stimulated by phorbol esters presumably via a protein kinase C-dependent pathway (Akhurst et al 1988), and by TGF- β 1 itself (Van Obberghen-Schilling et al 1988). The 5' region of the TGF- β 1 gene contains two transcription start sites (Kim et al 1989a); one promoter site located upstream of the first transcriptional start site and a second located between the two start sites (Kim et al 1989b), as well as several transcriptional inhibitory regions (Kim et al 1989a, 1990). Both promoters contain transcriptional enhancer elements that respond to induction by phorbol esters and TGF- β 1, or transactivation by AP-1 (Kim et al 1990). Activation via these elements is mediated by binding of the (Jun-Fos) AP-1 complex (Kim et al 1990). Additional putative phorbol ester responsive elements are present in the 3' flanking region of the TGF- β 1 gene (Scotto et al 1990). Since expression and activity of *jun* and *fos* genes are modulated by numerous factors including their own products (Sassone-Corsi et al 1988; Schütte et al 1989) as well as TGF- β 1 (Pertovaara et al 1989), these mechanisms have the capacity to finely tune TGF- β 1 expression in response to diverse stimuli.

With the exception of platelets, where TGF- β is stored in α -granules (Assoian & Sporn 1986), the TGFs- β appear to be released from cells via a constitutive secretory pathway. TGF- β 1, however, is released from either platelets or cultured cell lines as part of an inactive complex unable to

interact with cell surface receptors (Lawrence et al 1985; Pircher et al 1986). Exposure to extreme pH (<4 or >9), chaotropic agents (such as dodecyl sulfate, urea) or plasmin in vitro release active TGF- β from the latent complex (Lawrence et al 1985; Lyons et al 1988). The latent complex isolated from human platelets and fibroblasts consists of the mature TGF- β 1 dimer plus two TGF- β 1 pro-region polypeptides disulfide-linked to a glycoprotein of 125–160 kd in platelets, or 170–190 kd in fibroblasts (Miyazono et al 1988; Wakefield et al 1988; Kanzaki et al 1990). The pro-region polypeptides are also disulfide-linked to each other. The amino acid sequence deduced from the 125–160 kd glycoprotein cDNA contains multiple EGF-like repeats in tandem as the main distinctive feature (Kanzaki et al 1990). The function of this protein is unknown at the moment. This protein does not prevent binding of activated TGF- β 1 to cells, has no detectable proteolytic activity, and does not appear to bind activated TGF- β 1, or to be related to the TGF- β -binding proteoglycan, betaglycan (see below).

Studies with cells that overexpress a transfected TGF- β 1 gene, however, indicate that association of mature TGF- β 1 with the pro-region is sufficient to retain this factor in the latent state (Gentry et al 1988). Glycosylation and dimerization of newly translated TGF- β 1 precursor are followed by cleavage of the mature domain that continues to interact with the pro-region after release from the cell (Gentry et al 1988). The pro-region appears to be essential for the correct folding of TGF- β 1 during synthesis (Gray & Mason 1990). The TGF- β 1 pro-region contains mannose 6-phosphate (Purchio et al 1988) as well as the arg-gly-asp (RGD) sequence that in fibronectin, vitronectin, laminin, and other cell adhesion molecules recognizes certain adhesion receptors of the integrin class (Ruoslahti & Pierschbacher 1987). The TGF- β 1 pro-region can bind to cell surface mannose 6-phosphate receptors (Kovacina et al 1989), but it is not known whether the RGD sequence can mediate binding of proTGF- β s to integrins, or whether binding mediated by RGD or mannose 6-phosphate can lead to activation of latent TGF- β 1. The precise mechanisms that activate latent TGF- β in vivo are also unknown. Endothelial cell cultures can activate latent TGF- β , but only when cells are in contact with vascular pericytes (Antonelli-Olridge et al 1989; Sato & Rifkin 1989). Evidence suggests that the proteolytic action of plasmin or cathepsin D on the TGF- β 1 pro-region (Lyons et al 1988; Sato & Rifkin 1989), the removal of carbohydrate residues in this region (Miyazono & Heldin 1989), and the action of acidic microenvironments in sites of wound healing and bone resorption might contribute to activate latent TGF- β 1 in vivo.

Once released from the latent complex, active TGF- β 1 can be bound by various extracellular matrix components and serum proteins. Clearance

of circulating activated TGF- β is extremely rapid (<3 min; Coffey et al 1987) and binding to α_2 -macroglobulin might be involved in this process (O'Connor-McCourt & Wakefield 1987). TGF- β can accumulate in interstitial matrices (Thompson et al 1989). High affinity binding of TGF- β to the core protein of the proteoglycan, betaglycan (Andres et al 1989), or lower affinity interactions with abundant matrix components, might protect TGF- β from degradation, or might function as a long-term reservoir, sustained release mechanism, or TGF- β clearance system.

Inhibins and Activins

Inhibins and activins are dimeric polypeptides initially isolated from ovarian follicular fluid based on their ability to modulate the production of follicle-stimulating hormone (FSH) from pituitary cells (Ling et al 1985). The inhibins are composed of an α subunit and either a βA subunit (inhibin A), or βB subunit (inhibin B) (Mason et al 1985; Forage et al 1986). These heterodimers inhibit production of pituitary FSH, gonadal sex steroids, and placental hormones (Mason et al 1985; Forage et al 1986; Ying et al 1986b; Petraglia et al 1989). The amino acid sequences of the two β subunits are 60% identical. The α subunit sequence is as divergent from the β subunits (~25% identity at the C-terminal domain) as it is from the other members of the TGF- β family. There is substantial evidence indicating that inhibins are indeed physiologic regulators of FSH production (Rivier et al 1986). Inhibins appear to repress FSH production by decreasing FSH- α and FSH- β mRNA levels (Attardi et al 1989).

In addition to dimerizing with the α subunit, βA and βB chains can pair with each other (Vale et al 1986; Ling et al 1986). Remarkably, the resulting dimers $\beta A\text{-}\beta A$ and $\beta A\text{-}\beta B$ have biological activity opposite to that of inhibins. These dimers, called activins, stimulate FSH production by pituitary cell cultures, steroidogenesis in granulosa cells, and production of gonadotropin-releasing hormone, chorionic gonadotropin, and progesterone in cultured human placenta cells (Vale et al 1986; Ling et al 1986; Petraglia et al 1989). Furthermore, some of these actions are mimicked by TGF- $\beta 1$ (Ying et al 1986a). The actions of inhibins and activins are not restricted to gonadal and pituitary cells. Homodimeric βA activin was independently identified as a factor produced by human leukemia cells capable of inducing differentiation of erythroleukemia cells (Eto et al 1987). This action is antagonized by inhibins but, in contrast to other actions, it is not mimicked by TGF- $\beta 1$ (Eto et al 1987; Yu et al 1987). More recently, expression of α , βA , and βB subunits has been detected in many other extragonadal tissues (Meunier et al 1988). Remarkably, activin A is produced by *Xenopus laevis* XTC cells in culture and has potent

mesoderm-inducing activity in mid-blastula *Xenopus* embryo explants (Smith et al 1990).

The antagonistic activity of inhibins and activins raises questions concerning their mode of action. Do these factors oppose each other by acting through receptors that produce signals of opposite sign, or do they compete antagonistically for the same receptors? Activins are antagonized not only by inhibins, but also by follistatin, an activin-binding protein found in follicular fluid and other tissues (see below). Thus, as in the case of TGF- β , complex mechanisms appear to ensure a tight control of the activity of these factors.

Decapentaplegic, Vg1, and BMPs

The biology of these TGF- β -related factors underscores the role of certain members of this family as morphogens in arthropod and vertebrate developmental processes. The decapentaplegic (DPP) gene complex encodes important functions in embryonic as well as larval *Drosophila* pattern formation. Mutations in various regions of the DPP gene complex result in failed dorsal-ventral patterning during early embryogenesis and defective patterning of the larval imaginal disks (Spencer et al 1982). The mutations affect *cis*-regulatory elements that control the expression of a set of overlapping transcripts. The product encoded by these transcripts has the predicted structure of a TGF- β -related molecule, with a C-terminal sequence that is 36% identical to the mature TGF- β 1 sequence (Padgett et al 1987).

Another member of the family, Vg1, is involved in embryonic development in *Xenopus laevis*. Vg1 is encoded by a maternally inherited mRNA that is restricted to the vegetal (endodermal) pole of the embryo (Weeks & Melton 1987). The Vg1 product is ~38 and ~50% identical, respectively, to TGF- β 1 and DPP. The presence of maternal Vg1 mRNA persists through the process of mesoderm induction, in which it may participate, and declines sharply after gastrulation (Melton 1987). Vgr-1, a mouse cDNA isolated by its homology to Vg1, encodes a product whose predicted C-terminal domain is roughly as similar (~60% identity) to the corresponding sequence in the Vg1 product as to the DPP product and to BMP-2 (Lyons et al 1989). Vgr-1 expression in the mouse increases throughout development and into adulthood in many tissues and is induced during endodermal differentiation of F9 teratocarcinoma cells.

Bone has a unique capacity for self-restoration. An activity extracted from bone, and designated bone morphogenetic protein (BMP), induces chemotactic, proliferative, and differentiative responses that culminate with the transient formation of cartilage followed by accumulation of bone

with hematopoietic marrow (Wozney 1989). This process is quite distinct from promotion of growth of preexisting bone, an action that can be induced by various growth factors and hormones including TGF- β 1. Purified BMP preparations contain multiple component polypeptides (Wang et al 1988). N-terminal sequencing and molecular cloning of these components has shown that with the exception of BMP-1, the BMPs are members of the TGF- β superfamily and are particularly similar to DPP and Vg1 (Wozney et al 1988; Wozney 1989). The predicted amino acid sequences of human and bovine BMP-2 (formerly BMP-2A) and BMP-4 (formerly BMP-2B) show the highest degree of C-terminal domain identity (~75%) with *Drosophila* DPP, and they may represent the mammalian counterparts of this protein. BMP-5, 6, and 7 are closely related to each other and, like BMP-3 (also named osteogenin, Luyten et al 1989), show ~60% identity in their C-terminal domain with BMP-2 (Wozney 1989). BMP-2, 3, 6, and 7 have been isolated from human and/or bovine bone and are bioactive as TGF- β -like homodimers or heterodimers. BMP-4 and 5 are known only at the cDNA level. Physiologically, BMPs may act in concert with other factors to induce properly balanced bone formation. Recombinant BMP-2 implanted alone, however, is sufficient to induce ectopic bone production (Wang et al 1990).

Müllerian Inhibiting Substance

Müllerian inhibiting substance (MIS), also known as anti-Müllerian hormone, was identified and later purified based on its ability to induce regression of the primordium of female genitalia, the Müllerian duct, in mammalian male embryos (Blanchard & Josso 1974). MIS is produced by Sertoli cells of the fetal and adult testis and by ovarian granulosa cells after birth (Blanchard & Josso 1974; Vigier et al 1984). The deduced sequence of MIS C-terminal domain is ~25% identical to that of the other TGF- β -related factors. Purified from testes, MIS is a disulfide-linked homodimer of 70–74-kd glycosylated chains that, in contrast to the other TGF- β -related factors, contains the glycosylated N-terminal extension uncleaved from the C-terminal domain (Cate et al 1986). Recombinant MIS expressed in cultured cells, however, can undergo TGF- β -like proteolytic processing (Pepinski et al 1988). It is conceivable that the bioactive MIS entity could be generated by cleavage of the precursor at the sites of action *in vivo*.

In addition to its activity as a regulator of gonadal morphogenesis, MIS can act on ovarian endocrine differentiation. In organ culture, MIS induces endocrine sex reversal in fetal ovaries with release of testosterone instead of estradiol (Vigier et al 1989). This action appears to result from decreased

aromatase activity caused by MIS and correlates with the formation of seminiferous cord-like structures. Expression of MIS led to abnormal sexual development including the progressive degeneration of the ovaries in transgenic female mice, and overexpression of MIS led, paradoxically, to feminization and regression of genitalia in transgenic male mice (Behringer et al 1990). These observations suggest that MIS may promote testicular morphogenesis in addition to Müllerian duct regression. As with other TGF- β -related factors, progress in MIS research is revealing a broader range of actions than was originally anticipated.

BIOLOGICAL ACTIONS OF TGF- β

Most of the current information on the activity of TGF- β derives from the study of TGF- β 1, 2, and 3. As mentioned above, these TGF- β 1 isoforms acting on cultured cells often display similar activity and potency, but occasionally show marked differences (Ohta et al 1987; Ottmann & Pelus 1988; Tsunawaki et al 1988; Jennings et al 1988; Cheifetz et al 1990).

The ability of TGF- β to elicit multiple cellular responses, including responses of opposite sign, has been a subject of great interest. The paradigm of TGF- β as a dual factor emanated first from studies on cell proliferation; depending on the conditions, TGF- β can either inhibit or stimulate proliferation (Tucker et al 1984; Moses et al 1985; Roberts et al 1985; Massagué et al 1985). In some instances, the mechanism that leads to this duality is apparent. For example, TGF- β 1 action slows the cell cycle of AKR-2B mouse fibroblasts (Shipley et al 1985), but induces expression of platelet-derived growth factor-B (PDGF-B) in these cells (Leof et al 1986). Thus when AKR-2B cells are placed in a mitogen-rich medium, there is a net growth inhibitory effect of TGF- β 1, but when AKR-2B cells are plated in a mitogen-free medium, there is a net growth stimulatory effect caused by TGF- β 1-induced autocrine PDGF. In another example, TGF- β decreases the proliferation rate of NRK-49F rat fibroblasts cultured as monolayers in the presence of EGF (Roberts et al 1985), but allows these anchorage-dependent cells to grow in a semisolid medium by inducing the production of a fibronectin-collagen-proteoglycan extracellular matrix to which the cells can adhere (Ignatz & Massagué 1986; Bassols & Massagué 1988). Many of the diverse effects of TGF- β on cell proliferation and phenotype are less susceptible to simple explanations. As discussed below, important cell-specific determinants dictate the nature of a cell's response to TGF- β . Comprehensive accounts of the response of individual cell types to TGF- β are available (Roberts & Sporn 1990; Ignatz & Massagué 1990). The following summarizes some important aspects of the biological activity of the TGFs- β .

Control of Cell Proliferation

The concept that cell proliferation can be restrained not only by limitations in the supply of mitogenic stimuli, but also by the action of negative growth regulators (Holley et al 1980), has been substantiated by the identification of TGFs- β as some of the most potent growth inhibitors known to date (Tucker et al 1984). All TGF- β forms tested display reversible growth inhibitory activity in normal as well as transformed epithelial, endothelial, fibroblast, neuronal, lymphoid, and hematopoietic cell types (Tucker et al 1984; Moses et al 1985; Roberts et al 1985; Shipley et al 1986; Kehrl et al 1986a; Frater-Schröder et al 1986; Carr et al 1986; Cheifetz et al 1987, 1990; Knabbe et al 1987; Ohta et al 1987; Kimchi et al 1988; Graycar et al 1989). In certain cell lineages, TGF- β opposes the action of specific mitogens such as EGF in keratinocytes (Coffey et al 1988), IL-1 and IL-2 in lymphocytes (Ristow 1986; Kehrl et al 1986a,b; Wahl et al 1988), and IL-3, GM-CSF, and CSF-1 in hematopoietic progenitor cells at different stages of differentiation (Ohta et al 1987; Keller et al 1988). The extent of the growth inhibitory response to TGF- β varies with the cell type and reaches a virtual growth arrest in certain lung epithelial cells, lung fibroblasts and keratinocytes (Tucker et al 1984; Shipley et al 1986; Chambard & Pouyssegur 1988). TGF- β acts by lengthening or arresting the G1 phase of the cell cycle (Shipley et al 1985; Nakamura et al 1985; Heimark et al 1986; T. Lin et al 1987; Laiho et al 1990).

Evidence for an antiproliferative action of TGF- β 1 *in vivo* has been obtained with inert polymer beads impregnated with TGF- β 1 and implanted near the epithelial end buds of immature mammary glands (Silberstein & Daniel 1987). Also, intravenous injection of TGF- β 1 or 2 has a negative effect on the proliferative response of regenerating rat liver (Russell et al 1988). TGF- β 1 mRNA is expressed in terminally differentiating cells adjacent to the suprabasal layer of phorbol ester-treated epidermis, which suggests a role in epidermal cell withdrawal from the proliferative state (Akhurst et al 1988). TGF- β is expressed in apical cells from enteric villi but not in proliferative crypt enterocytes (Barnard et al 1989). The antiproliferative action of TGF- β on B-lymphocytes (Kehrl et al 1986a), T-lymphocytes (Kehrl et al 1986b), and thymocytes (Ristow 1986) is one of the components of the immunosuppressive activity that these factors display *in vitro* and *in vivo* (Wrann et al 1987; de Martin et al 1987) (see below).

TGF- β can also stimulate cell proliferation although, as mentioned above, the mitogenic effect may be secondary to other cellular responses. TGF- β stimulates proliferation of NRK-49F and AKR-2B fibroblasts plated in a semisolid medium (Roberts et al 1981; Moses et al 1981) or in

a mitogen-poor medium (Massagué 1984; Leof et al 1986), and of early human embryo fibroblasts (Hill et al 1986) and rat calvaria osteoblasts (Centrella et al 1987).

Control of Cell Adhesion

Cell migration, homing, and settlement during tissue formation, repair, tumor invasion, and metastasis are guided by a complex set of adhesive interactions between cells and extracellular matrices. Many of the cell surface components mediate adhesion of cells to the extracellular matrix and to other cells. The adhesive behavior of a cell is determined in part by the type and level of adhesion receptors that it expresses, and the type of extracellular matrix that it produces and with which it interfaces. In addition to providing physical support, adhesive interactions are a major conduit for intercellular regulation of cell function and phenotype. The possibility that the cell adhesion apparatus and the composition of extracellular matrices might be regulated by growth and differentiation factors is clearly apparent in the action of TGF- β on many cell types.

The action of TGF- β on normal mesenchymal, epithelial, and lymphoid cells, as well as various tumor cell lines, generally leads to up-regulation of cell adhesion. This action is mediated in concert by enhanced synthesis and deposition of extracellular matrix components, decreased pericellular proteolysis, and modification of the repertoire of cell surface adhesion receptors, as summarized in Figure 2. The original impetus to examine the expression of these molecules in response to TGF- β came from the observation of TGF- β effects on cell morphology and differentiation (Ignatz & Massagué 1985) and its involvement in wound healing, a process with intense accumulation and remodeling of the extracellular matrix (Roberts et al 1986). The marked and generalized effect of TGF- β on extracellular matrices is likely to play a major role in processes of organismal morphogenesis and development, tissue repair processes, and the pathogenesis of certain fibrotic diseases.

CONTROL OF EXTRACELLULAR MATRIX PROTEIN EXPRESSION TGF- β action elevates fibronectin expression in several mesenchymal and epithelial cell types, both normal and transformed (Ignatz & Massagué 1986; Like & Massagué 1986; Dean et al 1988). Up to tenfold elevation in fibronectin synthesis, and a corresponding increase in extracellular matrix fibronectin accumulation, are frequently observed in response to TGF- β 1 (Ignatz et al 1987; Dean et al 1988). TGF- β also regulates the expression of type I collagen α 1 and α 2 chains, and collagen types III, VI, and X (Ignatz & Massagué 1986; Roberts et al 1986; Varga et al 1987). Expression of type II collagen is induced in mesenchymal muscle cells secondarily to their

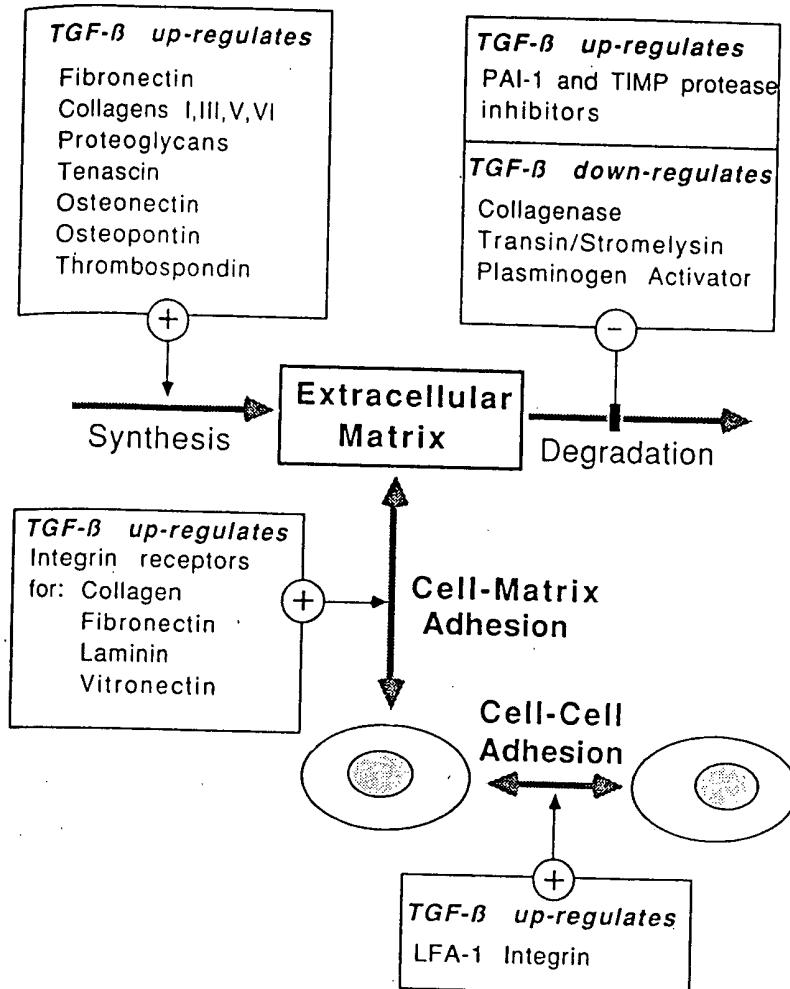


Figure 2 Cell adhesion molecules whose expression is regulated by TGF- β .

chondrogenic differentiation in response to TGF- β 1 and 2 (Seyedin et al 1985). Other matrix glycoproteins, whose synthesis is elevated in response to TGF- β 1, include osteopontin (Noda et al 1988), osteonectin (Noda & Rodan 1987), tenascin (Pearson et al 1988), thrombospondin (Penttinen et al 1988), and the chondroitin/dermatan sulfate proteoglycans, biglycan (PG I) and decorin (PG II) (Bassols & Massagué 1988).

Elevated levels of mRNA for these proteins are observed within 3–5 hr

of TGF- β addition and the consequence of elevated transcription of the corresponding genes, as well as increased mRNA stability in response to TGF- β (Ignatz et al 1987; Raghow et al 1987; Rossi et al 1988; Penttilä et al 1988; Dean et al 1988). The relative contribution of these two mechanisms may vary with the cell type. Not only do TGF- β 1 and 2 elevate the expression of proteoglycan core proteins, but they also increase the size or total mass of glycosaminoglycan (GAG) chains attached to them (Bassols & Massagué 1988). The composition of GAG chains in the mouse epithelial cell membrane proteoglycan syndecan (Rapraeger 1989), and the overall synthesis of glycosaminoglycans in arterial smooth muscle cells, skin fibroblasts, and growth plate chondrocytes are also up-regulated by TGF- β (Chen et al 1987; Falanga et al 1987). The mechanism by which TGF- β affects the elongation and termination of GAG chains is presently unknown.

The expected net effect of TGF- β action is an accumulation of extracellular matrix. Indeed, this is a major response observed following local injection of TGF- β 1 in animals (Roberts et al 1986). In addition, the action of TGF- β may also lead to important qualitative changes in the composition and structure of extracellular matrices. For example, an alteration in the relative proportion of fibronectin and tenascin may lead to changes in cell behavior because of the antagonistic role of these two proteins as mediators of cell adhesion (Chiquet-Ehrismann et al 1988).

CONTROL OF PERICELLULAR PROTEOLYSIS Elevated synthesis of extracellular matrix components is not solely responsible for the net accumulation of extracellular matrix induced by TGF- β . Plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitor of metalloprotease (TIMP), two inhibitors of extracellular matrix degrading enzymes, are strongly up-regulated by TGF- β (Laiho et al 1986, 1987; Lund et al 1987; Edwards et al 1987). The up-regulation of PAI-1 mRNA is due, at least in part, to increased transcription, occurs with faster kinetics (<30 min) than the response of other extracellular matrix components, and can reach up to 50-fold over the basal level. In addition, TGF- β can also decrease the expression of collagenase (Edwards et al 1987), transin/stromelysin (Matsrian et al 1986; Kerr et al 1988), plasminogen activator (Laiho et al 1986), and a thiol proteinase (Chiang & Nilsen-Hamilton 1986).

CONTROL OF CELL ADHESION RECEPTORS The integrins are one of the best characterized families of cell adhesion receptors (Hynes 1987; Ruoslahti & Pierchsbacher 1987). Integrins were examined in detail as targets for TGF- β action after it was observed that TGF- β -treated cells have a higher ability to bind fibronectin and collagen (Ignatz & Massagué 1986). Mouse

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thymocytes treated with TGF- β bind more readily to fibronectin-coated tissue culture dishes, and their attachment can be prevented by short synthetic RGD-containing peptides, which indicates that adherence is mediated by integrins (Ignatz & Massagué 1987). Integrins are heterodimeric membrane glycoproteins that consist of one 130–200-kd α subunit and one 90–130-kd β subunit. At least four distinct β integrin subunits exist in human cells, each one able to pair with various α subunits that confer ligand-binding preference to the resulting $\alpha\beta$ integrin complex. Many integrins function as adhesion receptors for extracellular matrix components including fibronectin, collagen, laminin, vitronectin, fibrinogen, von Willebrand factor, and other as yet unidentified matrix components. Integrins that have a $\beta 2$ subunit function as cell-cell adhesion receptors in lymphocytes. Most cell types express various integrins simultaneously, each cell type having a characteristic complement of these receptors.

TGF- β induces marked alterations in the repertoire of integrins expressed in many cell types, primarily by increasing the mRNA levels of individual subunits (Ignatz & Massagué 1987; C. J. Roberts et al 1988; Heino et al 1989; Ignatz et al 1989). TGF- β can alter the expression of all integrin subunits examined to date. These include (a) α_1 through α_6 subunits and the β_1 subunit, which combine to generate receptors for fibronectin, collagen, laminin, and other extracellular matrix molecules; (b) α_v and β_3 subunits that form a vitronectin receptor; and (c) the α_L subunit that combines with β_2 to constitute LFA-1, a cell-cell adhesion receptor that binds to intercellular adhesion molecule ICAM-1 on the surface of other lymphoid cells. Correct assembly of integrins is required for their expression on the cell surface. If some subunits are available in limited amounts, the unassembled excess of other subunits is degraded even before they transit through the Golgi apparatus. Since several integrins may share a common pool of β subunits, the relative change in cell surface integrin levels induced by TGF- β depends not only on the changes in the rate of synthesis of individual subunits, but also on the balance of the various α and β subunits produced by the cell (Heino et al 1989).

The susceptibility of individual integrins to up-regulation by TGF- β depends on the cell type (Heino et al 1989). The expression of a given integrin subunit may not be elevated in a given cell type, or may even be strongly down-regulated, as is the case of the α_3 subunit in MG-63 osteosarcoma cells (Heino & Massagué 1989). Through this set of up- and down-regulatory events TGF- β can alter the repertoire of cell adhesion receptors and the ability of cells to interact with other cells and extracellular matrices.

Control of Cell Phenotype

Initial reports of TGF- β 1 influencing cellular differentiation processes (Ignatz & Massagué 1985; Seyedin et al 1985; Masui et al 1986) and the expression of differentiated functions (Rook et al 1986; Hotta & Baird 1986) led to studies showing that the differentiative potential of many cell lineages can be affected by TGF- β in vitro. Table 2 includes a summary of cell lines or primary cell cultures whose differentiation is regulated, positively or negatively, by TGF- β . In addition, the table lists various specialized functions of terminally differentiated cells that are affected by these factors.

General features of the inhibitory action of TGF- β on cell differentiation have been defined in studies on preadipocytes and myoblasts. Mouse 3T3-L1 preadipocytes (Green & Meuth 1974) and rat L₆E₉ skeletal muscle myoblasts (Nadal-Ginard 1978) can be induced to differentiate, respectively, into mature adipocytes and multinucleated myotubes. Differentiation becomes complete within 3–4 days after induction. When these cells are induced to differentiate in the presence of picomolar concentrations of TGF- β , differentiation is blocked (Ignatz & Massagué 1985; Massagué et al 1986; Florini et al 1986). TGF- β 1 can be added as late as 30 hr post-induction and still block preadipocyte differentiation. Once 3T3-L1 cells become committed to differentiate, however, they are refractory to the inhibitory action of TGF- β 1. Like preadipocytes, L₆E₉ myoblasts go through a critical temporal point after which differentiation will proceed even in the presence of TGF- β . This refractoriness to TGF- β is not due to a loss of receptors since 3T3-L1 adipocytes and L₆E₉ myotubes continue to express TGF- β receptors I and II at levels similar to those of the undifferentiated counterparts, and they continue to show various biochemical responses to TGF- β , such as elevation of extracellular matrix protein expression (Ignatz & Massagué 1985; Massagué et al 1986). Inhibition of differentiation is not secondary to effects of TGF- β 1 on cell proliferation, and it is reversible once TGF- β 1 is removed (Ignatz & Massagué 1985; Massagué et al 1986; Florini et al 1986). The inhibitory effect of TGF- β on 3T3-L1 and L₆E₉ cell differentiation correlates with a marked alteration in the expression of extracellular matrix proteins and cell adhesion receptors (Ignatz & Massagué 1986, 1987; Ignatz et al 1987). The possible participation of this response in mediating the anti-differentiative action of TGF- β is discussed below.

TGF- β is thought to favor chondrogenesis and osteogenesis, and it exerts positive effects on these cell lineages in vitro (Seyedin et al 1985; Pfeilschifter et al 1987); however, TGF- β can inhibit the expression of osteogenic differentiation markers in some cell lines (Noda & Rodan 1987;

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Table 2 Effects of TGF- β 1 on cell phenotype

Cell type ^a	Function	Effect of TGF- β ^b	References
Preadipocytes (3T3-L1, Balb/c 3T3, TA1)	Differentiation	-	Ignotz & Massagué 1985; Sparks & Scott 1986; Torti et al 1989
Skeletal muscle myo- blasts (L ₆ , L ₈ , C2, BC, H1)	Differentiation	-	Massagué et al 1986; Olson et al 1986; Florini et al 1986
Muscle satellite cells	Differentiation	-	Allen & Boxhorn 1987
Prechondroblasts	Differentiation	+	Seyedin et al 1985
Osteoblastic osteosarcoma (ROS 17/2.8)	Differentiation	+	Pfeilschifter et al 1987
Osteoblasts (MC3T3L1)	Differentiation	-	Noda & Rodan 1987;
Intestinal epithelial cells (IEC-6)	Differentiation	+	Rosen et al 1988
Megakaryocytes	Differentiation	-	Kurokawa et al 1987;
Hematopoietic progenitor cells (B6Sut-A, 32D-cl3)	Differentiation associated with proliferation	-	Barnard et al 1989
Bronchial epithelial cells	Differentiation markers	+	Ishibashi et al 1987
Natural killer cells	Cytolysis	-	Ohta et al 1987;
B-lymphocytes	Ig production	-	Keller et al 1988;
Lymphocyte-activated killer cells	Cytokine production	-	Ottmann & Pelus 1988
Monocytes	Cytokine production	+	Masui et al 1986;
Macrophages	Respiratory burst	-	Jetten et al 1986
Endothelial cells	Invasion	-	Rook et al 1986
Keratinocytes	Keratinogenesis	+	Kehrl et al 1986a
Adrenocortical cells	Steroidogenesis	-	Espevik et al 1988
Leydig cells	Steroidogenesis	-	Wahl et al 1987
Granulosa cells	Steroidogenesis	+	Tsunawaki et al 1988
Pituitary cells	FSH production	+	Müller et al 1987
Adipocytes (TAI-1)	Lipogenesis	-	Reiss & Sartorelli 1987;
			Mansbridge & Hanawalt 1988
			Hotta & Baird 1986;
			Feige et al 1987
			Linn et al 1987;
			Avallet et al 1987
			Ying et al 1986a
			Ying et al 1986b
			Torti et al 1989

^aContinuous cell lines are indicated in parentheses. ^b-, inhibition; +, stimulation.

Rosen et al 1988). These distinct responses might reflect genuine differences between osteogenic cell types. Alternatively, they might reflect a distortion of the normal TGF- β response by the constraints of in vitro culture conditions. Of course, the latter consideration also applies to the other

differentiative processes that are affected by TGF- β in vitro. In vitro systems may demonstrate the existence of biochemical mechanisms that couple TGF- β to the control of cell phenotype, but in vivo studies are necessary to establish the response that activation of these mechanisms by TGF- β will ultimately induce in the physiologic setting.

Physiology and Pathology

EMBRYOGENESIS TGFs- β are expressed throughout embryogenic development, and their receptors are ubiquitously distributed. This, and the ability of TGF- β to control DNA replication, cell differentiation, cell adhesion, and extracellular matrix layout suggest a broad role for TGF- β in the generation and modification of extracellular cues that guide the morphogenic events of embryogenesis. Thus far, however, the evidence that TGFs- β play a critical role in embryogenesis remains correlative in nature.

Expression of TGFs- β is high in sites undergoing intense development and morphogenesis. These include sites of chondrogenesis and osteogenesis: long bone growth plates or somites developing into vertebrae (Ellingsworth et al 1986; Heine et al 1987; Sandberg et al 1988), hematopoietic organs (Ellingsworth et al 1986; Wilcox & Derynck 1988), and epithelial/mesenchymal interfaces (Lehnert & Akhurst 1988; Pelton et al 1989). That early embryo tissues are responsive to TGF- β has been demonstrated by addition of mammalian TGF- $\beta 1$ (together with fibroblast growth factor) or TGF- $\beta 2$ to ectoderm explants from *Xenopus laevis* embryos. These additions promote the generation of mesoderm (Kimmelman & Kirschner 1987; Rosa et al 1988).

TISSUE REPAIR, INFLAMMATION, AND ANGIOGENESIS TGFs- β stored at high levels in platelets (Assoian et al 1983; Cheifetz et al 1987) or expressed in activated monocytes and macrophages (Assoian et al 1987; Tsunawaki et al 1988) can be physiologically delivered to sites of wound healing or inflammation. The constant remodeling and recycling of bone matrix and marrow, or their repair, are accessible to control by TGFs- β that are abundantly present in these tissues (Seyedin et al 1985; Thompson et al 1989). The activity of TGF- β as a promoter of extracellular matrix deposition and a regulator of cell migration and development probably plays a major influence in these processes. In addition, TGF- $\beta 1$ is an extremely potent chemoattractant for monocytes (Wahl et al 1987) and, to a lesser extent, fibroblasts (Postlethwaite et al 1987). TGF- $\beta 1$ may attract these cells to sites of inflammation and repair. Indeed, administration of TGF- $\beta 1$ into wound chambers, subcutaneously, or to incisional wounds, stimulates the accumulation of granulation tissue and cellularization of the

wound bed and accelerates the wound healing response in general (Sporn et al 1983; Roberts et al 1986; Mustoe et al 1987).

Directly or through other cells that it attracts and stimulates, TGF- β 1 can induce formation of new blood vessels in vivo (Roberts et al 1986). This response might seem paradoxical given the strong growth inhibitory effect of TGF- β 1 and TGF- β 3 on endothelial cell monolayer cultures (Frater-Schröder et al 1986; Heimark et al 1986; Cheifetz et al 1990). It has been noted, however, that under certain culture conditions endothelial cells are not growth-inhibited by TGF- β 1 and tend to organize into tubular structures reminiscent of an angiogenic process (Majack 1987; Madri et al 1988).

IMMUNOSUPPRESSION The observation that glioblastoma is frequently accompanied by immunosuppression led to the isolation of TGF- β 2 as a glioblastoma cell-derived suppressor of T cells in vitro (Wrann et al 1987; de Martin et al 1987). Both TGF- β 1 and TGF- β 2 exhibit activities in vitro that are consistent with an immunosuppressive action in vivo. These include the antiproliferative effects on thymocytes, T- and B-lymphocytes described above, as well as multiple negative effects on differentiated functions of B-lymphocytes, natural killer cells, lymphocytes activated killer cells, and macrophages, as described in Table 2 and references therein. The importance of the TGF- β contribution to the pathophysiology of immunosuppression remains to be determined.

FIBROSIS A localized excess of TGF- β activity in tissues could lead to an unbalanced deposition of extracellular matrix and contribute to a variety of fibrotic disorders. A case in point is the condition known as proliferative vitreoretinopathy (PVR) (Connor et al 1989). PVR occurs in 10% of eyes that undergo surgery for retinal detachment, and it leads to excessive intraocular fibrosis and blindness. The levels of TGF- β activity in PVR are threefold higher than normal; this activity appears to correspond to TGF- β 2. Injection of TGF- β into the vitreous cavity of test animals produces a PVR condition (Connor et al 1989). Detailed studies of other fibrotic disorders might reveal a wider role of TGF- β in these disorders.

ONCOGENESIS Unrestricted cell growth caused by the lack of TGF- β growth inhibitory activity is perhaps the most important of the possible consequences that would derive from a pathological loss of TGF- β function. Such loss could be caused by defects in TGF- β expression or activation, or defects at the TGF- β receptor or post-receptor levels. The level of TGF- β expression varies widely among normal cell lines, however, and many normal cell lines are unable to activate the latent TGF- β that they produce in vitro. These facts generate some uncertainty in assessing

to what extent a low level of expression or activation of TGF- β in a given tumor-derived cell type might contribute to the oncogenic transformation of these cells. Similar limitations exist in determining the significance of a lack of growth inhibitory response to TGF- β in tumor cells since not all normal cells are growth inhibited by these factors. It is worth noting, however, that rat liver epithelial cells (McMahon et al 1986) and human retinoblastoma cells (Kimchi et al 1988) do not respond to TGF- β , whereas their normal counterparts are growth inhibited by this factor.

The loss of TGF- β receptors is a highly unusual event among some 150 cell lines and primary cells examined, normal or transformed (Wakefield et al 1987; Massagué et al 1990). Human retinoblastoma cells are a notable exception to the ubiquitous distribution of TGF- β receptors (Kimchi et al 1988). Eight independently derived retinoblastoma cell lines are not growth inhibited by TGF- β and lack detectable TGF- β receptors I or II (see below), in contrast to normal fetal retina cells, which display the full repertoire of TGF- β receptors and are growth inhibited by this factor. It has been suggested that the lack of functional TGF- β receptors in retinoblastoma cells, whose primary defect lies in the RB gene, may permit these tumor cells to escape growth suppression by TGF- β in the retina (Kimchi et al 1988). It remains to be determined how widespread the absence of TGF- β receptors is among other tumor cell types that have a defective RB gene locus, and which, if any, mechanism might link RB function to expression of TGF- β receptors. Other cell lines that have no TGF- β receptors I or II detectable by affinity-labeling techniques include PC12 rat pheochromocytoma cells and N2A mouse neuroblastoma cells (Kimchi et al 1988).

TGF- β RECEPTORS AND OTHER BINDING PROTEINS

Hormonally active polypeptides are believed to act on target cells by binding avidly and specifically to integral membrane proteins that are coupled to cytoplasmic signal transducers. TGF- β binds with high affinity to the surface of many cell types (Wakefield et al 1987). Covalent tagging of cells with radiolabeled TGFs- β using cross-linking agents or a photo-reactive TGF- β 1 analogue reveals that binding is mediated by several coexisting membrane proteins (Massagué & Like 1985; Cheifetz et al 1986). Some of these proteins are likely to mediate TGF- β action, whereas others may fulfill a different function.

Two glycoproteins (receptors I and II) of 53 and 70–100 kd, respectively, and a membrane proteoglycan, designated betaglycan (formerly type III

receptor) have been identified as the most widespread high-affinity TGF- β -binding components. Receptors I and II bind TGF- β 1 with higher affinity than betaglycan, and are ubiquitously present at low levels in mammalian and avian cells, with the exceptions mentioned above. Betaglycan is broadly distributed, but it is not detectable in various cell types that respond to TGF- β including skeletal muscle myoblasts, hematopoietic progenitor cells, and vascular endothelial cells (Massagué et al 1986, 1990; Ohta et al 1987; Segarini et al 1989; Goodman & Majack 1989). Properties of these TGF- β -binding components are listed in Table 3. A detailed review of their cellular distribution and other properties has been recently presented (Massagué et al 1990).

Isolation of TGF- β receptor cDNAs has been an elusive goal. Therefore, formal identification of the TGF- β receptor based on expressing its cDNA in receptor-defective cells has not yet been achieved. The cellular distribution of TGF- β receptors and, in particular, the isolation of TGF- β -resistant cell mutants, however, have provided substantial evidence implicating receptor types I and II as components of the signal-transducing TGF- β receptor complex.

TGF- β Receptors

RECEPTOR COMPONENTS I AND II The possibility that receptors I or II are involved in mediating TGF- β action was first suggested by the fact that TGF- β 1 controls cell differentiation, collagen, and fibronectin expression in skeletal muscle myoblasts that express receptors I and II, but no beta-

Table 3 Cell surface TGF- β -binding proteins

	Type I	Type II	Betaglycan	GH ₃ protein
Probable function	Signal transduction	Signal transduction	TGF- β storage or transport	Unknown
Ligands	TGFs- β	TGFs- β	TGFs- β	TGFs- β , activins inhibins
Glycoprotein, kd ^a	53 (65) ^b	70–100 (85–110)	200–400	60 (70–74)
Deglycosylated, kd ^c	48	55–75	100–120	60
Carbohydrate type ^c	N-linked	N-linked	Glycoaminoglycans and N-linked glycans	Unknown
K _D for TGF- β 1	5–50 pM	5–50 pM	30–300 pM	90 pM
Order of affinities	β 1 ~ β 3 > β 2	β 1 > β 3 > β 2	β 1 ~ β 2 ~ β 3	β 1 > β 2 ≥ Act ~ Inh
Binding sites/cell	Up to 4,000	Up to 4,000	Up to 10 ⁵	2,700
Distribution	Ubiquitous	Ubiquitous	Broad	GH ₃ , pituitary cells

^a Estimated molecular weight of the membrane glycoprotein form. ^b Molecular weight of the affinity-labeled complex is shown in parentheses. ^c Deduced by treatment of the protein with trifluoromethanesulfonic acid, N-glycanase, O-glycosidase, chondroitinase ABC and/or heparitinase.

glycan (Massagué et al 1987) and inhibits proliferation of murine hematopoietic progenitor cells expressing receptor type I as the only detectable TGF- β -binding component (Ohta et al 1987).

In all avian and mammalian cell types examined, the type I TGF- β receptor component is a glycoprotein of 53 kd, a value estimated by subtraction of the mass contributed by reduced TGF- β to the 65 kd affinity-labeled receptor complex (Cheifetz et al 1986). This receptor component contains an N-linked carbohydrate, which is not required for cell surface expression of the receptor, or for TGF- β binding, as shown after inhibition of co-translational glycosylation with tunicamycin (Cheifetz et al 1988a). Characteristically, the type I receptor discriminates between various forms of TGF- β in receptor competition assays. The order of relative affinities is TGF- β 1 ~ TGF- β 3 > TGF- β 1.2 > TGF- β 2, with a 10–20 fold difference between the affinity constants for TGF- β 1 and TGF- β 2 (Cheifetz et al 1987, 1988b, 1990; Segarini et al 1987).

The type II receptor shares many of the characteristics of the type I receptor. It is also a glycoprotein with N-linked carbohydrate that is not required for cell surface expression or for ligand binding (Cheifetz et al 1986, 1987, 1988a). This receptor can discriminate between the various forms of TGF- β with a similar, albeit not identical, range of affinities as the type I receptor. Despite these similarities, comparative peptide mapping experiments indicate that the binding domains of these receptors are distinct. Unlike the type I receptor, the type II receptor shows a great variability in size, from an estimated 70 to 85 kd in most mammalian cells to 100 kd in chick embryo fibroblasts (Cheifetz et al 1986). The size variability in mammalian cells appears to be largely the result of differences in glycosylation. However, the deglycosylated polypeptide from chick embryo fibroblasts is 20 kd larger than the corresponding mammalian product (S. Cheifetz, personal communication).

Receptor I is expressed in most of the over one hundred cell lines and tissues tested by affinity-labeling procedures (Massagué et al 1990). Several murine hematopoietic progenitor cell lines (B6SUt-A, 32D-C13) that are growth-inhibited by TGF- β are among the very few known cases in which no clearly detectable type II receptor is co-expressed with the type I receptor (Ohta et al 1987). These cells respond to TGF- β 1, 1.2, and 2 with an order of potencies that parallels the order of affinities of these factors for receptor I (Ohta et al 1987; Cheifetz et al 1988b). Human and bovine vascular endothelial cells, which express receptors I and II, are also growth-inhibited more potently by TGF- β 1 than TGF- β 2 (Jennings et al 1988), but in this case the difference appears to result from a more rapid inactivation of TGF- β 2 (Cheifetz et al 1990). Most other mammalian cell lines tested show similar sensitivity to various forms of TGF- β , whether the

cells co-express receptors I and II alone or with betaglycan. Recent binding saturation studies with Mv1Lu mink lung epithelial cells have suggested the presence of a population of receptors I and II that has high affinity for TGF- β 1, 2, and 3 and binds these factors in the concentration range that is sufficient for a maximal growth inhibitory response. The other, more abundant, receptor population has the order of affinities TGF- β 1 > TGF- β 3 > TGF- β 2 observed in receptor competition experiments using higher ligand concentrations (Cheifetz et al 1990). The biological role of these spare receptors is unknown.

EXPERIMENTALLY INDUCED RECEPTOR MUTANTS The phenotype of various experimentally induced TGF- β -resistant cell mutants derived from Mv1Lu cells provides the most persuasive evidence to date implicating TGF- β receptors I and II in signal transduction (Boyd & Massagué 1989; M. Laiho et al submitted). Parental Mv1Lu cells are potently ($ED_{50} = 1\text{pM}$) growth arrested by TGF- β and express receptors I and II, as well as betaglycan. The mutant cell clones have lost all known TGF- β responses including growth inhibition. With high frequency the mutations affect receptors I and II; however, betaglycan is not affected (Boyd & Massagué 1989; Laiho et al 1990). Some mutant clones show normal binding to the type II receptor, but are defective in type I receptor (R mutants), whereas other clones (S mutants) express all receptors in a profile identical to the parental cell line. A third group of mutants is defective in both the type I and type II receptors (DR mutants), which are either undetectable or present in very low levels with the type II receptor and have, in addition, decreased electrophoretic mobility. Analysis of somatic cell hybrids has indicated that all these mutant phenotypes are recessive (Boyd & Massagué 1989; M. Laiho et al submitted). The DR phenotype could be due to independent mutational events affecting both receptors, but a more attractive possibility is that receptors I and II interact with each other so that mutations affecting the expression or structure of one receptor component may impair the expression or function of the other.

A TGF- β RECEPTOR MODEL The phenotype of these TGF- β -resistant cell mutants implicates receptors I and II as components of the receptor complex that mediates multiple TGF- β responses including growth inhibition, extracellular matrix protein up-regulation, and the morphological response. Since receptors I and II are also present in cell lines that are not growth-inhibited by TGF- β , the different responsiveness of cells to these factors is probably determined by the ability of the receptor to couple with different signaling pathways in each cell type. A model in which a single receptor complex with two TGF- β -binding subunits mediates multiple actions of TGF- β prevails at present over the hypothesis that the different

responses to TGF- β mediated by multiple unrelated receptors. Given that TGFs- β are dimeric, it is possible that a single TGF- β molecule could simultaneously interact with the two receptor components I and II.

TGF- β RECEPTOR REGULATION Loss of individual TGF- β receptors in retinoblastoma cells (Kimchi et al 1988) suggests the existence of mechanisms that regulate TGF- β receptor expression. The available evidence, however, suggests that the expression and dynamics of TGF- β receptors are in general not highly regulated. TGF- β receptors do not appear to display the acute regulation by homologous and heterologous ligands that is commonly seen in receptors for some other growth factors. This is despite the fact that receptor-bound TGF- β is rapidly internalized and degraded (Frolik et al 1984; Massagué & Kelly 1986). TGF- β binding increases during differentiation of murine embryonal carcinoma cells (Rizzino 1987) and during T cell activation (Kehrl et al 1986b). Adrenocortical cells show increased TGF- β binding to all three receptor forms following stimulation with adrenocorticotropic hormone (Cochet et al 1988). Loss of betaglycan, or gain of betaglycan with loss of receptors I and II have been described, respectively, in smooth muscle cells and vascular endothelial cells as a function of cell density (Goodman & Majack 1989; Müller et al 1987). Other investigators, however, have not observed effects of cell density on TGF- β receptors in vascular endothelial cells (Segarini et al 1989). Changes in receptor profile as a function of cell density do not appear to be a general phenomenon (S. Cheifetz, personal communication).

Betaglycans: Proteoglycans with High Affinity for TGF- β

In many cell lines, the most abundant cell-surface TGF- β -binding component is betaglycan (Massagué 1985; Massagué & Like 1985; Cheifetz et al 1986; Fanger et al 1986). Betaglycan is heterogeneous in nature and typically runs on SDS-polyacrylamide gels as a broad band with an average mass of 280–330 kd. One of the remarkable characteristics of betaglycan is that it is an integral membrane proteoglycan consisting of approximately 200 kd of glycosaminoglycan (GAG) chain mass and 10 kd of N-linked glycans attached to a heterogeneous core polypeptide of 100–120 kd (Segarini & Seyedin 1988; Cheifetz et al 1988a). Betaglycan may contain only one class of glycosaminoglycan chains, but most betaglycan forms contain heparan sulfate as well as chondroitin sulfate GAG chains in a proportion that varies in different cell types (Cheifetz & Massagué 1989). In some cell lines, betaglycan appears to be part of a disulfide-linked complex (Massagué 1985; Fanger et al 1986). In contrast to receptors I and II, betaglycan shows similar affinity for TGF- β 1, 2, and 3 in many cell lines

tested (Cheifetz et al 1987, 1988b, 1990; Cheifetz & Massagué 1989), but one study suggests the presence of a mixed receptor population with different affinities for TGF- β 1 and TGF- β 2 in some cell lines (Segarini et al 1987).

Experiments with cell mutants defective in GAG synthesis (Cheifetz & Massagué 1989) and the use of GAG-degrading enzymes (Segarini & Seyedin 1988; Cheifetz et al 1988a) have shown that the TGF- β binding site resides in the betaglycan core protein and that the GAGs are not required for TGF- β binding or functional expression of betaglycan on the cell surface. The growth inhibitory response to TGF- β is similar in parental cells and the GAG-defective mutants.

Betaglycan may belong to the recycling receptor class since it appears to be internalized with TGF- β , but this does not lead to an acute down-regulation of cell surface betaglycan level (Massagué & Kelly 1986). Betaglycan forms that lack the membrane anchor are released by cultured cells into the medium and are found in low amounts in serum and in extracellular matrices (Andres et al 1989). The soluble forms of betaglycan also differ from the membrane forms in the electrophoretic mobility of their 100–110 kd core devoid of GAG chains. It is not known whether the soluble form of betaglycan is derived from the membrane bound form by a hydrolytic process, is the product of alternative mRNA splicing, or is encoded by a separate gene.

Betaglycan was the first TGF- β -binding component to be identified as a putative TGF- β receptor based on its relative abundance in 3T3 fibroblasts and the correlation between biological potency and betaglycan affinity for various TGF- β forms (Massagué & Like 1985; Cheifetz et al 1987). The TGF- β signal transducing activity is now ascribed to receptors I and II. Betaglycan might, however, be involved in ligand presentation to these receptors. Given its structural features, relative abundance, and secretory nature, betaglycan could function as a reservoir or clearance system for bioactive TGF- β . By analogy with membrane proteoglycans that may participate in cell adhesion and recognition (Rapraeger 1989), it is possible that betaglycan could also have such adhesive functions.

Activin-Binding Proteins

Betaglycan and the TGF- β receptors I and II do not recognize activins or inhibins. A TGF- β binding protein that cross-reacts with inhibins and activins, however, has been identified by affinity-labeling of GH₃ rat pituitary tumor cells (Cheifetz et al 1988c). This protein has an estimated molecular weight of 60 kd (after subtracting the mass of cross-linked TGF- β monomer). The affinity of this receptor for TGF- β 1 ($K_d = 90$ pM) is higher than for TGF- β 2, activin, or inhibin. These values parallel the

relative potency of these factors to regulate FSH production in rat pituitary cell cultures (Ling et al 1986; Ying et al 1986a). The presence of this receptor in primary pituitary cells could not be determined, and its biological significance remains to be determined.

Activin receptors ($K_d = 30 \text{ pM}$, 3,200/cell) are present in murine FS-5 erythroleukemia cells (Hino et al 1989). Binding of activin-A to these receptors is not inhibited by TGF- $\beta 1$, which is in agreement with the inability of TGF- $\beta 1$ to mimic activin action on erythroblastic cells (Eto et al 1987). Cross-linking of these receptors with radiolabeled activin yields two major labeled products of 67 and 76 kd, respectively; a pattern that is reminiscent of that for the affinity-labeled TGF- β receptors I and II. This resemblance raises the possibility that receptors for the activins and inhibins, the TGFs- β and, perhaps, the other factors of this large family have structural features in common.

An activin-binding protein isolated from rat ovary by activin-affinity chromatography is follistatin (Nakamura et al 1990). Follistatin is a soluble 35-kd glycoprotein that was initially identified by its ability to inhibit FSH production by the pituitary gland. Follistatin binds activin with high affinity ($K_d = 590 \text{ pM}$) and blocks its FSH-inducing activity in the pituitary (Nakamura et al 1990). Thus the action of activin appears to be tightly regulated by two types of antagonists, the inhibins and follistatin.

MECHANISM OF TGF- β ACTION

Based on the structural and functional homologies of the members of the TGF- β superfamily, it is likely that these factors interact with a family of structurally related receptors. The unique range of biological activities displayed by these factors suggests the possibility that their signal transduction mechanisms might be quite distinct from others presently known.

The Cytoplasmic Response

The primary signal transduction mechanism of TGF- β receptors is not known. Several efforts to determine whether enzymatic activities and second messengers that are directly involved in signaling by other hormone and growth factor receptors might be coupled to TGF- β receptors have met with negative results (Like & Massagué 1986; Chambard & Pouyssegur 1988; Fanger et al 1986; J. Massagué, unpublished work). Interestingly, agents that increase cAMP accumulation block induction of *c-sis* by TGF- $\beta 1$ in vascular endothelial cells (Daniel et al 1987). Inhibition of TGF- $\beta 1$ -induced *c-sis* expression by cholera and pertussis toxins has been reported in AKR-2B fibroblasts (Murthy et al 1988; Howe & Leof 1988; Howe et al 1990). These toxins catalyze ADP-ribosylation and inactivation of

guanine nucleotide-binding proteins (G proteins), which are involved in coupling certain hormone receptors to effector molecules. Both toxins can inhibit the mitogenic response of AKR-2B fibroblasts to TGF- β 1, but do not alter other TGF- β responses or TGF- β 1 binding to receptors (Howe et al 1990).

Activation of glycolysis, amino acid uptake, intracellular calcium levels, and phosphatidyl inositol turnover have been observed in rat fibroblasts in response to TGF- β (Boerner et al 1985; Inman & Colowick 1985; Muldoon et al 1988). The effect of TGF- β 1 on these parameters does not show the rapid kinetics characteristic of the response to certain hormones and pharmacologic agents. Furthermore, the elevation of cytosolic calcium and accumulation of inositol triphosphate appear to require gene transcription (Muldoon et al 1988), which indicates that these cytoplasmic responses are not directly coupled to TGF- β receptors. TGF- β 1 can also stimulate prostaglandin E₂ production in lung fibroblasts (Diaz et al 1989) and cultured mouse calvaria (Tashjian et al 1985).

The Nuclear Response

A basis for the diversity, sometimes disparity, of the biological responses induced by TGF- β in different cell types, or in the same cell type under different conditions, can be gleaned from studies on the control of gene expression by these factors. The expression of an array of genes related to growth control, differentiation, and cell adhesion can be markedly altered within 0.5 to 3 hr of addition of TGF- β to cells. Although post-transcriptional events (Raghow et al 1987; Penttinen et al 1988; Morrone et al 1989) may contribute to this response, TGF- β frequently controls gene expression at the transcriptional level, either positively (Ignotz et al 1987; Dean et al 1988; Lund et al 1987; Rossi et al 1988; Machida et al 1988; Kerr et al 1988; Thompson et al 1988; Kim et al 1989a, 1990) or negatively (Kerr et al 1988, 1990; Pietenpol et al 1990). The nature of the nuclear response to TGF- β is quite complex, but the accumulating evidence leads to the following conclusions.

First, TGF- β can regulate different transcriptional control elements. Furthermore, different TGF- β -responsive elements can coexist in the same gene promoter. For example, transcriptional activation by TGF- β 1 is mediated by a NF-1 binding site in the collagen α 2(I) gene promoter (Rossi et al 1988), by multiple AP-1 binding sites in the TGF- β 1 gene promoter (Kim et al 1990), and by three distinct elements in the fibronectin gene promoter. One of the elements in the fibronectin gene promoter is a NF-1 binding site; its deletion results in only a partial decrease in transcriptional activation by TGF- β (D. C. Dean, S. Bourgeois, personal communication).

Second, cell-specific determinants dictate the type of response of a par-

ticular gene to TGF- β . For example, transcription of *c-jun* and *junB* can be elevated by TGF- β , but whether one or both of these genes will respond, and with what kinetics, depends on the cell type (Pertovaara et al 1989; Kim et al 1990; Li et al 1990). A survey of numerous integrins has shown that expression of most of them can be elevated by TGF- $\beta 1$ action, but only a subset of the integrins expressed in a given cell type may respond to this factor (Heino et al 1989; Ignotz et al 1989). Expression of the α_3 integrin subunit in MG-63 osteosarcoma cells is inhibited by TGF- $\beta 1$ even though TGF- $\beta 1$ elevates expression of this integrin subunit in other cell types and elevates expression of other integrins in MG-63 cells (Heino & Massagué 1989). These responses appear to be at the transcriptional level (M. Bosenberg et al, unpublished), and it will be interesting to determine how TGF- β controls positive or negative regulatory elements in each cell type.

Third, some of the genes whose expression is regulated by TGF- β encode transcription factors (*c-jun*, *junB*, *fos*, *myc*), growth factors (PDGF-A, PDGF-B), or other products that can, in turn, generate a secondary nuclear response. Several of the proliferative and differentiative effects of TGF- β probably follow from this secondary wave of nuclear events.

Growth Suppression Mechanisms

Various mechanisms have been proposed to explain the growth inhibitory action of TGF- β . Several reports have tentatively attributed this action to the ability of TGF- β to decrease expression of mitogen receptors, e.g. EGF receptors in NRK rat fibroblasts (Assoian 1985), or PDGF receptors in bone marrow fibroblasts and 3T3 fibroblasts (Bryckaert et al 1988; Gronwald et al 1989). These effects may contribute to the growth-inhibitory response in some cell types, but evidence argues that they do not represent a general mechanism of growth inhibition by TGF- β . Thus EGF-induced proliferation of lung epithelial cells and lung fibroblasts is arrested by TGF- $\beta 1$ without altering EGF binding or an array of EGF-induced growth-related responses including activation of plasma membrane H⁺/Na⁺ antiport, induction of *c-fos* and *c-myc* expression, activation of protein kinase C, or phosphorylation of ribosomal protein S6 (Like & Massagué 1986; Chambard & Pouyssegur 1988 and personal communication). One report proposed that inhibition of NRK fibroblast proliferation is secondary to the action of collagen overproduced in response to TGF- $\beta 1$ (Nugent & Newman 1989). Accumulation of a collagen-rich extracellular matrix in response to TGF- $\beta 1$ may affect the long term proliferative capacity of these cells, but the rate of collagen accumulation in fibroblasts is too slow to mediate rapid effects on the cell cycle.

Other approaches have focused on identifying cell cycle events that are directly controlled by TGF- β action. TGF- β 1 has been shown to inhibit the expression of certain growth-related genes, particularly *c-myc*, in endothelial cells, colon carcinoma cells, keratinocytes, and breast carcinoma cells (Takehara et al 1987; Fernandez-Pol et al 1987; Coffey et al 1988; Mulder et al 1988). TGF- β 1 inhibits *c-myc* gene transcription in mouse keratinocytes stimulated by EGF (Coffey et al 1988; Pietenpol et al 1990). This effect is involved in the growth inhibitory response of keratinocytes to TGF- β (Pietenpol et al 1990). No negative response of *c-myc* has been observed in fibroblasts whose proliferation is blocked by TGF- β 1 (Chambard & Pouyssegur 1988; Sorrentino & Bandyopadhyay 1989). Although TGF- β 1 can increase the expression of *c-fos*, *c-jun* and *jubB*, these responses do not appear to correlate with effects on cell proliferation (Pertovaara et al 1989; Kim et al 1990; Heino & Massagué 1990).

TGF- β inhibits cell cycle progression by lengthening or arresting the G1 phase (Shipley et al 1985; Nakamura et al 1985; Heimark et al 1986; P. Lin et al 1987). In Mv1Lu lung epithelial cells, TGF- β 1 acts by interfering with late G1 events (Laiho et al 1990). Significant among these events is phosphorylation of the retinoblastoma gene product, RB. The retinoblastoma gene encodes a product with presumptive growth suppressor activity (Dryja et al 1985; Friend et al 1986; Fung et al 1987; Lee et al 1987). In normal cells, RB is expressed throughout the cell cycle, but exists in multiple phosphorylated forms that are specific for certain phases of the cycle (Ludlow et al 1989; DeCaprio et al 1989; Buchkovich et al 1989; Chen et al 1989). It is thought that the cell cycle regulatory (suppressive) activity of RB is regulated by cell cycle-dependent phosphorylation and dephosphorylation events (Buchkovich et al 1989; DeCaprio et al 1989; Ludlow et al 1990). Underphosphorylated RB is the form with presumed growth suppressive activity since it is prevalent in G1 and in growth-arrested cells (DeCaprio et al 1989; Buchkovich et al 1989; Chen et al 1989) and is selectively bound by the transforming protein of SV40 virus T antigens which might inactivate it (Ludlow et al 1989).

Studies based on these new insights have shown that TGF- β 1 added to lung epithelial cells in mid to late G1 rapidly prevents the phosphorylation of RB scheduled for this time point and arrests cells in cycle (Laiho et al 1990). Furthermore, expression of T antigen in Mv1Lu cells does not prevent the effect of TGF- β on RB phosphorylation, but prevents the growth inhibitory response presumably by blocking the growth suppressive activity of unphosphorylated RB. The extent of inhibition of RB phosphorylation induced by TGF- β 1 in several cell lines (A549 lung adenocarcinoma, MG-63 osteosarcoma, BSC-1 monkey kidney epithelial cells) is proportional to the intensity of their growth inhibitory response to TGF-

628 These findings suggest that TGF- β 1 and RB function in a common growth inhibitory pathway in which TGF- β 1 acts to retain RB in the underphosphorylated, growth suppressive state.

Differentiation Control Mechanisms

The events that commit cells to terminal differentiation have begun to be defined. A family of myogenic differentiation genes, including *MyoD1*, *myogenin*, *Myf-5* and *myd* (Davis et al 1987; Pinney et al 1988; Wright et al 1989; Braun et al 1989), are expressed at high levels during the differentiation process, and induce cell commitment to a muscle phenotype. The *MyoD1*, *myogenin* and *Myf-5* products have a domain with predicted homology to the helix-loop-helix motif of Myc. This structure is implicated in protein dimerization (Davis et al 1987; Wright et al 1989; Braun et al 1989), in binding to certain muscle gene enhancers (Lassar et al 1989a), and in transcriptional activation of muscle-specific genes (Weintraub et al 1989). Expression of *myogenin* differentiation genes might be the subject of regulation by factors that control myogenic differentiation, such as TGF- β 1. Indeed, TGF- β 1 has been shown to repress transcription of *MyoD1* in 23A2 mouse myoblasts (Vaidya et al 1989) and to prevent elevation of *myogenin* mRNA in L₆E₉ rat myoblasts (Heino & Massagué 1990). These responses may relate to the ability of TGF- β 1 to up-regulate Jun expression since overexpression of v-Jun (P. Vogt & H. Su personal communication) or its associated protein, Fos (Lassar et al 1989b), are incompatible with myogenic gene expression.

Given the prominent role that *myogenin* plays in myoblast differentiation (Wright et al 1989), the ability of TGF- β 1 to prevent up-regulation of *myogenin* mRNA is likely to participate in the inhibition of L₆E₉ myoblast differentiation by this factor; however, additional mechanisms may be involved in the anti-myogenic action of TGF- β 1. This possibility is suggested by the observation that fibroblasts rendered competent to differentiate by forced expression of *MyoD1* or *myogenin* fail to differentiate in the presence of TGF- β 1 (Vaidya et al 1989; Heino & Massagué 1990). The nature of the alternative mechanism has been suggested by the observation that L₆E₉ myoblasts respond to TGF- β 1 with a marked elevation of type I collagen synthesis and deposition in the matrix (Massagué et al 1986; Ignotz et al 1987), and L₆E₉ myoblasts plated on a collagen-rich matrix do not differentiate even though their *myogenin* mRNA level increases several-fold (Heino & Massagué 1990). Cell adhesion is a determinant of L₆ myoblast differentiation, as shown by the ability of an anti-integrin antibody to block this differentiation process (Menko & Boettiger 1987). Thus inhibition of myoblast differentiation by TGF- β 1 appears to be accomplished by two mechanisms acting in concert. One of these mech-

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anisms leads to a block in the expression of a myogenic differentiation gene, such as *myogenin* in L₆E₉ cells. The other mechanism is likely to involve TGF- β -induced changes in cell adhesion that either block the action of myogenic differentiation gene products or prevent the function of other as yet unknown components of the myogenic differentiation pathway.

PROSPECTS

The TGF- β gene superfamily undoubtedly will be more complex than we presently know it. Systematic searches for TGF- β -related factors as well as serendipitous findings will soon outdate the information in Table 1. Current work is also aimed at gaining an understanding of the mechanisms that regulate expression of the various TGF- β -related factors, control activation of their latent forms, and their storage and clearance after release. This information is required to exploit the complex biology of these factors for therapeutic purposes. The spectrum of unique actions of the TGFs- β suggests a plethora of potential useful applications in medicine, but more knowledge and a better understanding of the biological and chemical properties of these factors is needed to use them effectively.

Some of the major tasks that lie ahead relate to the elucidation of the mechanisms of action of TGF- β -related factors and the determinants of cellular responsiveness to them. How can TGF- β regulate diverse transcriptional control factors and orchestrate a distinct pleiotropic nuclear response in each cell type? If, as the emerging evidence indicates, the components that drive the cell cycle are so remarkably conserved in organisms from yeast to humans, how can cells vary so much in their proliferative response to TGF- β ? Are the differences dictated by unique sets of biochemical components that couple TGF- β receptors to gene expression and cell cycle control mechanisms in each cell type? The identification of the primary structures of the TGF- β receptors is likely to be accomplished soon. This should help identify the signal transduction components, and components beyond these, in the pathway of TGF- β action. We anticipate a great deal of excitement as the study of the TGFs- β , one of the most fascinating networks of intercellular communication by polypeptide factors, unfolds.

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Regulation of left-right patterning in mice by growth/differentiation factor-1

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The transforming growth factor- β (TGF- β) superfamily encompasses a large group of structurally related polypeptides that are capable of regulating cell growth and differentiation in a wide range of embryonic and adult tissues¹. Growth/differentiation factor-1 (*Gdf1*, encoded by *Gdf1*) is a TGF- β family member of unknown function that was originally isolated from an early mouse embryo cDNA library² and is expressed specifically in the nervous system in late-stage embryos and adult mice³. Here we show that at early stages of mouse development, *Gdf1* is expressed initially throughout the embryo proper and then most prominently in the primitive node, ventral neural tube, and intermediate and lateral plate mesoderm. To examine its biological function, we generated a mouse line carrying a targeted mutation in *Gdf1*. *Gdf1*^{-/-} mice exhibited a spectrum of defects related to left-right axis formation, including visceral *situs inversus*, right pulmonary isomerism and a range of cardiac anomalies. In most *Gdf1*^{-/-} embryos, the expression of *Ebf1* (formerly *lefty-1*) in the left side of the floor plate and *Leftb* (formerly *lefty-2*), *Nodal* and *Pitx2* in the left lateral plate mesoderm

was absent, suggesting that *Gdf1* acts upstream of these genes either directly or indirectly to activate their expression. Our findings suggest that *Gdf1* acts early in the pathway of gene activation that leads to the establishment of left-right asymmetry. Northern blot analysis of whole embryo RNA using a *Gdf1* probe detected two developmentally regulated transcripts, a 1.4-kb transcript containing the *Gdf1* coding region, which was expressed at early embryonic stages, and a bi-cistronic 3.0-kb transcript, which was expressed in later embryonic stages and in the nervous system of adult mice⁴ (Fig. 1a). To examine the distribution of the 1.4-kb *Gdf1* transcript, we performed whole-mount *in situ* hybridization experiments on early stage mouse embryos. At 7.5 days post-coitum (d.p.c.), we detected *Gdf1* mRNA uniformly throughout the embryo proper, but not in extra-embryonic structures (Fig. 1b,c). By 8.0–8.5 d.p.c., we saw *Gdf1* mRNA in many tissues including the crown of the primitive node, ventral neural ectoderm and paraxial, intermediate and lateral plate mesoderm (Fig. 1d–g). At all stages examined, the expression pattern of *Gdf1* appeared to be bilaterally symmetric with respect to the left-right axis.

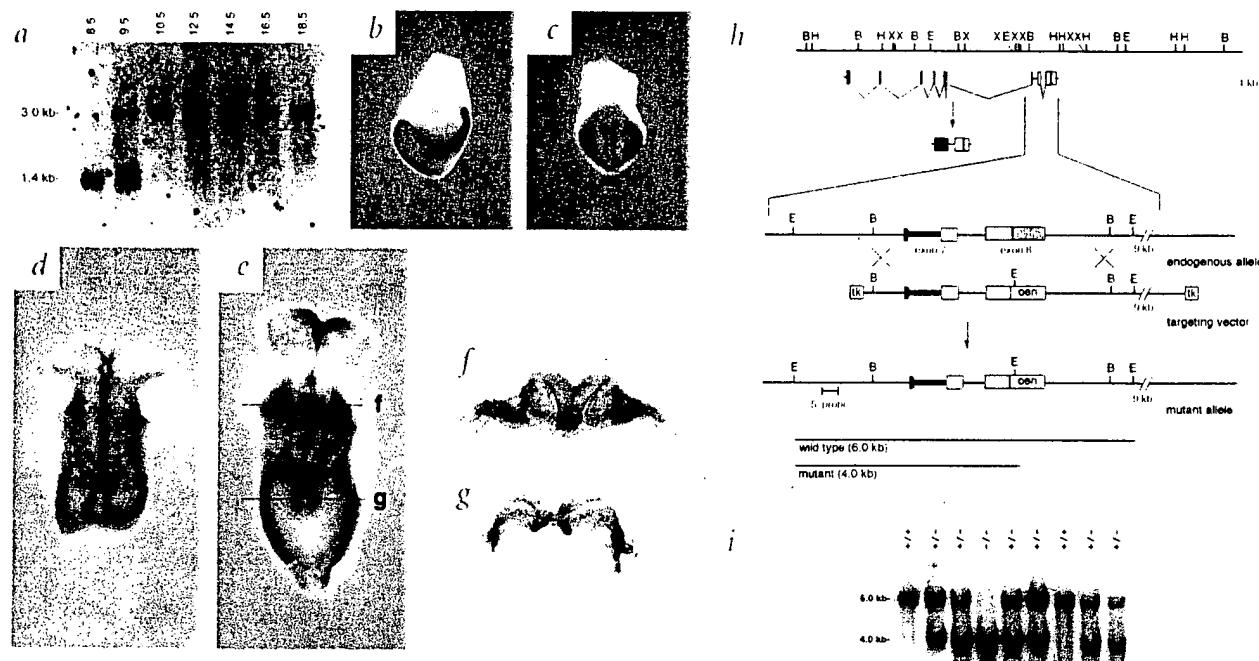


Fig. 1 *Gdf1* expression during early mouse development. **a**, Northern blot analysis of whole embryo RNA. Poly(A)⁺-selected mRNAs prepared from CD-1 mouse embryos at the indicated d.p.c. were electrophoresed, blotted and probed with the entire *Gdf1* coding sequence. **b**–**c**, Whole-mount *in situ* hybridization analysis of 7.5 d.p.c. (**b**, lateral view; **c**, anterior view), 8.0 d.p.c. (**d**) and 8.5 d.p.c. (**e**) mouse embryos. Expression is symmetric at all stages examined. **f**–**g**, Sections through a 8.5 d.p.c. embryo at the levels shown in (**e**). **h**, Genomic map of the *Gdf1* locus and targeting strategy. Filled boxes represent the coding sequence for UOG-1 (ref. 3). Open and shaded boxes represent the coding sequences for the pro- and carboxy-terminal regions of *Gdf1*, respectively. A probe derived from genomic sequence upstream of the targeting construct hybridizes to a 6-kb *Eco*RI fragment in wild-type *Gdf1* and a 4-kb *Eco*RI fragment in a homologously targeted *Gdf1* allele. **i**, Genomic Southern blot of *Eco*RI-digested liver DNA prepared from newborn offspring of a mating of heterozygous mice.

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Table 1 • Defects in *Gdf1*^{-/-} newborn mice

	A	B	C	D	E	F	G	H	I
Heart malformation	✓	✓	✓	✓	✓	✓	✓	✓	✓
Right pulmonary isomerism	✓	✓	✓	✓	✓	✓	✓	✓	✓
GI tract, spleen and pancreas	normal	✓	✓	✓	✓	✓	✓	✓	✓
Liver	reversed	—	—	—	—	✓	✓	✓	✓
Kidneys	normal	✓	✓	—	—	—	—	—	—
	reversed	—	—	—	—	✓	✓	—	—
	symmetric	—	—	✓	✓	✓	—	✓	✓
Total		10	2	2	2	1	9	1	4
									3

Newborn *Gdf1*^{-/-} mice were classified into groups on the basis of their combinations of anatomical *situs* defects.

To investigate the biological function of *Gdf1*, we generated mice in which the entire region encoding the mature *Gdf1*-1 peptide was deleted by gene targeting (Fig. 1*h*). Among 481 offspring examined from *Gdf1*^{+/-} intercrosses, only one homozygous mutant survived to adulthood. *Gdf1*^{-/-} embryos appeared to be viable up to approximately 14.5 d.p.c. Only two-thirds of *Gdf1*^{-/-} embryos survived until birth, however, and nearly all of these died within the first 48 hours after birth. The cause of death was likely to be related to the presence of extensive cardiac defects, although we have not ruled out the possibility that other abnormalities in these mutants might also have contributed to their severely reduced viability.

Homozygous mutant mice exhibited a complex spectrum of abnormalities related to improper establishment of left-right asymmetry (Table 1 and Fig. 2). The most obvious manifestations were the placement of the abdominal organs, which appeared to be randomized in *Gdf1*^{-/-} mutants with respect to the left-right axis. Visceral *situs inversus* was most readily apparent with respect

to the stomach, which is normally positioned on the left side (Fig. 2*a–c*). In 50% of *Gdf1*^{-/-} mutants the stomach was positioned on the right side. In *Gdf1*^{-/-} mice with right-sided stomachs, the direction of rotation of the small and large intestines was also reversed (Fig. 2*d,e*, and data not shown). Hence, the gastrointestinal tract appeared to have a mirror-image configuration relative to the left-right axis in approximately 50% of *Gdf1*^{-/-} mice.

Abnormalities in left-right axis formation were evident in other abdominal organs as well. For example, the positions of the pancreas and spleen, which are normally left-sided (Fig. 2*b*), paralleled that of the stomach in *Gdf1*^{-/-} mutants (Fig. 2*c*). The defects in the development of these organs, however, were more complex, as many *Gdf1*^{-/-} mutants had an annular pancreas, and all had severely malformed spleens. Mutation of *Gdf1* also altered the relative positioning of the kidneys and adrenal glands, which are normally displaced caudally on the left side (Fig. 2*f*). In *Gdf1*^{-/-} mice, the left kidney and adrenal were positioned either more cranially than the right kidney and adrenal (Fig. 2*g*) or at the same rostral-caudal level. Similarly, the normal asymmetric arrangement of the liver was also disrupted in *Gdf1*^{-/-} mice. In wild-type mice, the liver consists of a large left lateral lobe, left and right medial lobes, and three smaller lobes on the right side. In *Gdf1*^{-/-} mice, the liver lobes appeared to be reversed with respect to the left-right axis or bilaterally symmetric, with two equally sized lateral lobes beneath a fused medial lobe.

We also saw aberrant left-right patterning of the heart and lungs in homozygous mutants, but the nature of the defects in the thoracic organs appeared to be independent of whether or not the abdominal organs exhibited *situs inversus*. Wild-type mice have five lung lobes, a single large lobe on the left side and four smaller lobes on the right side (Fig. 2*h*). In contrast, *Gdf1*^{-/-} mice had eight lung lobes that were symmetrically distributed with respect to the left-right axis (Fig. 2*i*). The duplication of the right-sided pattern, or right pulmonary isomerism, was observed in all homozygous mutants. The heart defects in *Gdf1*^{-/-} mice were much more complex and variable. In wild-type mice, the apex of the heart points towards the left side of the animal (Fig. 3*d–f*). In *Gdf1*^{-/-} mice, however, the position of the apex was randomized (Fig. 3*g–l*). All mutant hearts also showed abnormal positioning of the great vessels (Fig. 3*a–c*). The pulmonary artery (blue) normally exits the heart ventrally and to the right of the aorta (yellow). In contrast, the pulmonary artery in *Gdf1*^{-/-} mice was positioned more dorsally than the aorta,

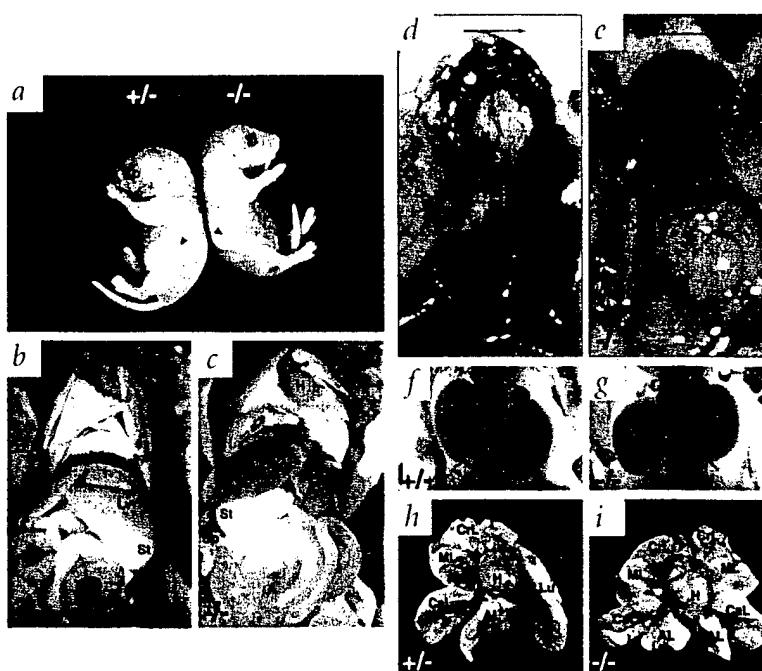
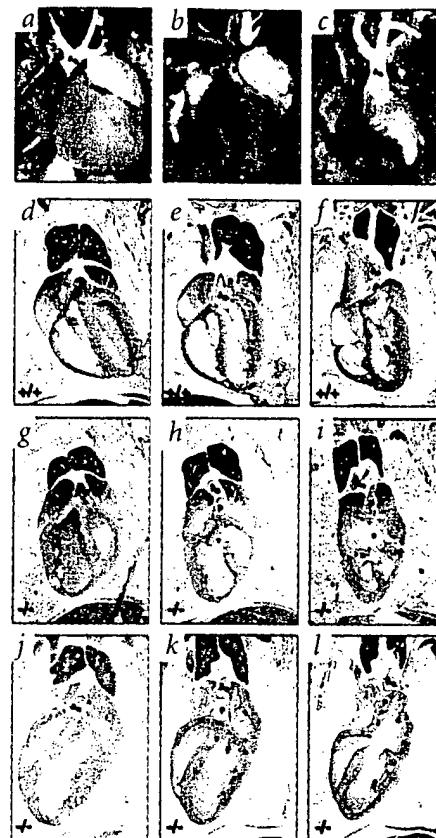


Fig. 2 Analysis of *situs* defects in *Gdf1*^{-/-} mice. **a**, *Gdf1*^{+/-} and *Gdf1*^{-/-} newborn mice with stomachs (arrowheads) on the left and right sides, respectively. Ventral views of tissues from newborn *Gdf1*^{+/-} (**b,d,f,h**) and *Gdf1*^{-/-} (**c,e,g,i**) mice are shown. **b,c**, Reversal of the orientation of the abdominal organs in *Gdf1*^{-/-} mice. Note also the streak-like appearance of the spleen and the abnormally shaped medial lobe of the liver. **d,e**, Reversal of the direction of rotation of the colon in *Gdf1*^{-/-} mice. **f,g**, Reversal of the rostral-caudal arrangement of the kidneys in *Gdf1*^{-/-} mice. **h,i**, Right pulmonary isomerism in *Gdf1*^{-/-} mice. **h**, heart; **Lv**, liver; **St**, stomach; **Sp**, spleen; **AC**, ascending colon; **DC**, descending colon; **RK**, right kidney; **LK**, left kidney; **Crl**, cranial lung lobe; **ML**, medial lung lobe; **CaL**, caudal lung lobe; **AL**, accessory lung lobe; **LLu**, left lung lobe.

Fig. 3 Heart defects in *Gdf1*^{-/-} mice. **a–c**, Dye injections of *Gdf1*^{+/+} (**a**) and *Gdf1*^{-/-} (**b,c**) mice. Yellow dye was injected into the ventricle positioned on the left side, and blue dye was injected into the ventricle on the right side. Note that the relative dorsal/ventral and or left/right relationships between the positions of the aorta and pulmonary artery are reversed in *Gdf1*^{-/-} mice. Frontal sections of wild-type (**d,e,f**) and two *Gdf1*^{-/-} (**g,h,i**, mesocardia; **j,k,l**, dextrocardia) hearts are shown. For each, three sections progressing from the ventral to the dorsal side are shown. In wild-type heart, the pulmonary artery exits the heart more ventrally than does the aorta. In *Gdf1*^{-/-} mice, the aorta exits more ventrally. Ventricular septal defects (**h,k,l**, arrowheads) and atrial septal defects (**i**, asterisk) were also seen in *Gdf1*^{-/-} mice.

although the left-right relationship of these arteries was randomized. In these mutants, the abnormal positioning of the aorta and pulmonary artery reflected a transposition of the great vessels. Histological examination of serial sections of five *Gdf1*^{-/-} hearts revealed additional defects as well, including atrial and ventricular septal defects, common atrioventricular canal and persistent left vena cava (Fig. 3g, and data not shown).

To examine the relationship of *Gdf1* with other genes implicated in left-right determination, we carried out whole-mount *in situ* hybridization on *Gdf1*^{-/-} embryos with probes directed against *Ebf1*, *Leftb* and *Nodal*, encoding TGF- β family members, as well as *Pitx2*, encoding a transcription factor (Table 2). In wild-type embryos at the headfold stage (~4–6 somites), *Ebf1* is predominantly expressed on the left side of the presumptive floor plate, and *Leftb* is mainly expressed in the left lateral plate mesoderm (LPM; Fig. 4a). Using a probe that detects both *Ebf1* and *Leftb* transcripts⁵, we were unable to detect expression of these genes in most (6/7) headfold-stage *Gdf1*^{-/-} embryos examined (Fig. 4b). Expression of *Nodal* in headfold-stage wild-type embryos is normally seen in the node and left LPM (Fig. 4c; refs 6,7). Although the expression of *Nodal* in the node remained unchanged, the expression of *Nodal* in the LPM was absent in all mutant embryos examined (Fig. 4d). *Pitx2*, which is normally present in the left LPM of wild-type embryos^{8,9} (Fig. 4e), was also downregulated in 8 of 11 *Gdf1*^{-/-} embryos (Fig. 4f). These results suggest that *Gdf1* is necessary for the induction or maintenance of the asymmetric expression of *Ebf1*, *Leftb*, *Nodal* and *Pitx2* in the early embryo.



Our findings indicate that *Gdf1* is essential for proper establishment of the left-right axis in mice. Although a number of other secreted proteins, including other members of the TGF- β superfamily, have been implicated as important regulators of left-right axis determination in vertebrates¹⁰, *Gdf1* is unusual in that loss of *Gdf1* function leads to complete visceral *situs inversus* in a large percentage of mutant animals. In this regard, the *Gdf1*^{-/-} phenotype more closely resembles that of mice carrying the *Dnahc11*^{iv}

Table 2 • Gene expression in *Gdf1*^{-/-} embryos

Gene	Genotype	Somites	Floor plate				Lateral plate mesoderm				Total
			left	right	bilateral	absent	left	right	bilateral	absent	
<i>Ebf1/Leftb</i>	+/+	0–4	1	–	–	3	–	–	–	4	4
		4–6	5	–	–	1	6	–	–	–	6
		6–8	–	–	–	1	1	–	–	–	1
	+/-	0–4	5	–	–	4	–	–	–	9	9
		4–6	10	–	–	1	9	–	–	2	11
		6–8	2	–	–	6	8	–	–	–	8
	-/-	0–4	–	–	–	4	–	–	–	4	4
		4–6	1	–	–	6	1	–	–	6	7
		6–8	–	–	–	6	1	–	–	5	6
<i>Nodal</i>	+/+	0–4	6	–	–	–	–	–	–	6	6
		4–6	8	–	–	–	8	–	–	–	8
		6–10	3	1	–	–	–	–	–	4	4
	+/-	0–4	9	–	–	–	–	–	–	9	9
		4–6	12	–	–	–	12	–	–	–	12
		6–10	6	2	–	–	1	–	–	7	8
	-/-	0–4	6	–	–	–	–	–	–	6	6
		4–6	3	–	–	–	–	–	–	3	3
		6–10	7	2	–	–	1	–	–	8	9
<i>Pitx2</i>	+/+	6–10	–	–	–	–	left	right	bilateral	absent	Total
		6–10	–	–	–	–	5	–	–	–	5
	+/-	6–10	–	–	–	–	23	–	–	5	28
		6–10	–	–	–	–	3	–	–	8	11

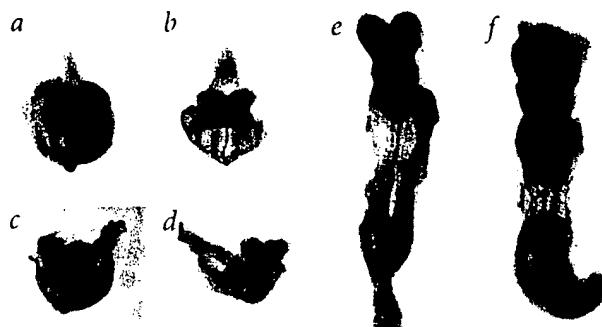


Fig. 4 Asymmetric gene expression in *Gdf1*^{-/-} mice. Embryos in (a-d) are viewed from the anterior; (e) and (f) are seen from the ventral side. **a**, Normal *Ebaf* and *Leftb* expression in the left LPM and floor plate. The expression of *Ebaf* and *Leftb* in the floor plate was verified by examining sections of these embryos. **b**, Absence of visible *Ebaf* and *Leftb* expression in *Gdf1*^{-/-} embryos. The midline staining in (b) represents background hybridization that did not correspond to the floor plate based on an analysis of sections of these embryos. **c**, Normal expression of *nodal* in the left LPM (node staining is obscured by the LPM staining). **d**, Absence of *nodal* expression in the node of *Gdf1*^{-/-} embryos. Note the normal expression of *nodal* in the node. **e**, Normal expression of *Pitx2* in the head mesenchyme and left LPM. **f**, Absence of *Pitx2* expression in the LPM of *Gdf1*^{-/-} embryos, whereas expression in the head mesenchyme remains normal.

allele^{11,12} and that of humans with certain asplenia syndromes that are characterized by *situs inversus*^{13,14}. *Gdf1* is also unusual in that its expression pattern during development appears to be symmetric with respect to the left-right axis. Although we cannot rule out the possibility that there is some asymmetric expression of *Gdf1* that is either very transient or below the level of detection in our experiments, our data suggest that the mechanism by which *Gdf1* influences left-right asymmetry must involve other molecules that are asymmetrically expressed, such as those responsible for generating biologically active Gdf-1 protein or for transducing the Gdf-1 signal. Alternatively, Gdf-1 protein may be synthesized in a symmetric pattern, but become asymmetrically distributed by some other mechanism. In this regard, it has been hypothesized that a leftward flow of fluid generated by cilia present in the node may be responsible for the directional movement of a morphogen produced by the node^{15,16}. Given the intense expression of *Gdf1* in the node and the finding that *Gdf1* acts upstream of *Ebaf*, *Leftb*, *nodal* and *Pitx2*, *Gdf1* may be a possible candidate for this morphogen. An elucidation of the mechanism of action of Gdf-1 will require a careful examination of the distribution of the mature Gdf-1 protein and the identification of molecules directly involved in Gdf-1 signalling, including the Gdf-1 receptor.

Methods

In situ hybridization and northern-blot analysis. We carried out hybridizations using a probe corresponding to the entire *Gdf1* coding region². We prepared the *Ebaf*, *Leftb* (ref. 5) and *nodal* (ref. 17) probes as described. The *Pitx2* probe was provided by M. Blum and M.R. Kuehn. Embryos were isolated from timed matings of CD-1 mice. We carried out northern-blot analysis using poly(A)-selected RNA (2 µg) as described². Whole-mount *in situ* hybridization analysis was carried out as described^{18,19}, except that 20% heat-inactivated sheep serum was used for antibody blocking and incubation steps. Sections (10–12 µm) of stained embryos were prepared using a cryostat.

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Construction and analysis of *Gdf1*-null mice. The structure of *Gdf1* was deduced from restriction mapping and partial sequencing of phage clones isolated from a mouse 129 Sv/J genomic library. Vectors for preparing the targeting construct were provided by P. Soriano and K. Thomas. RI embryonic stem cells (provided by A. Nagy, R. Nagy and W. Abramow-Newerly) were transfected with linearized targeting construct (50 µg) and selected with gancyclovir (2 µM) and G418 (200 µg/ml). Following electroporation of the targeting construct into embryonic stem cells, we identified homologous targeting by Southern blot analysis in 4 of 90 clones resistant to both G418 and gancyclovir. Examination of the *Gdf1*-mutant phenotype was carried out using offspring on a C57Bl/6/129SV/J hybrid background from a chimaera derived from blastocyst injection of one of these clones. Genomic Southern blots were carried out as described²⁰. We genotyped embryos by PCR using genomic DNA isolated from extra-embryonic membranes²¹. Primers for genotyping were as follows: *Gdf1* wild-type allele, 5'-GTTGCG GCTGGAGGCTGAGAG-3' and 5'-CCCACTGGACCAACTCTACC-3'; *Gdf1* targeted allele, 5'-CCCACTGCAGCCTGTGGCGC-3' and 5'-GGAA GACATAGCAGGCATGCTGG-3'.

For analysis of heart morphology, we performed latex dye injections as described²². Casting dyes (Connecticut Valley Biological Supply) were injected into the ventricles of the heart using a pulled capillary glass pipette. For histological analysis of *Gdf1*-mutant hearts, we killed newborn mice and infused them with Bouin's fixative through the trachea. The entire animal was then fixed by immersion in Bouin's fixative for at least 24 h. Mouse chests were step-sectioned (5 µm sections, 10 µm between each section) from the exit of the aorta or pulmonary artery to their respective branch points dorsally. For analysis, we stained sections with haematoxylin and eosin.

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